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Departamento de Ciências Médicas

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Molecular mechanisms in obesity and intensive training in children

Mecanismos moleculares na obesidade e treino intensivo em crianças

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro e co-orientação do Professor Doutor Fernando Manuel Tavares da Silva Ribeiro, Professor Adjunto da Escola Superior de Saúde da Universidade de Aveiro.

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O júri

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Palavras-chave

Obesidade infantil, exercício intenso, inflamação, perfil lipídico, tecido adiposo, músculo esquelético, índice de massa corporal.

Resumo

A inatividade física é um dos principais riscos para a obesidade. Esta doença crónica resulta de um desiquilíbrio calórico causando um alargamento dos adipócitos através do excesso de armazenamento de gordura. Com um aumento da prevalência, a obesidade infantil correlaciona-se com a disfunção endotelial, inflamação e stress oxidativo, conduzindo ao desenvolvimento de outras doenças não só em criança, mas também durante a idade adulta. Por outro lado, muitas crianças praticam exercício de elevada duração ou intensidade em desportos de alta competição, o que pode ter efeitos prejudiciais a nível físico, fisiológico e psicológico. Em jovens atletas de alta competição, stress oxidativo e imunossupressão podem ocorrer levando ao elavado risco de infeção. No entanto, perfis lipídicos melhorados são encontrados em crianças atletas. Desta forma, o objetivo do presente estudo foi analizar o impacto da obesidade infantil bem como de treinos intensivos de natação na composição corporal, inflamação e perfil lipídico através de análises ao sangue, bioimpedância e imunodeteção de citocinas pró-inflamatórias (IL-6 e TWEAK), uma miocina (Miostatina) e uma proteina de fase aguda (CRP). Para tal, foram recrutados 24 jovens divididos em três grupos: obesos, atletas e normoponderais. O grupo de obesos apresentou elevados níveis de gordura corporal, um perfil lipídico atípico (baixo HDL e elevado LDL), níveis elevados de lactato desidrogenase no sangue indicando dano tecidual, inflamação crónica (elevado IL-6, CRP e TWEAK) e massa muscular diminuida (elevada Miostatina) sem dano muscular (baixo CK). No entanto os baixos níveis de enzimas hepáticas (AST e ALT) no soro não associam a obesidade com doença hepática. Por outro lado, o exercício físico intenso não é uma atividade prejudicial para os jovens atletas, uma vez que o perfil lipídico é melhorado e o aumento dos níveis de marcadores inflamatórios não é significativo. O principal benefício do treino intensivo é a diminuição dos níveis de glucose tendo um papel protetor para a diabetes.

Keywords Childhood obesity, intensive exercise, inflammation, lipid profile, adipose tissue, skeletal muscle, body mass index.

Abstract

Physical inactivity is a major risk for obesity. This chronic disease results from a caloric imbalance causing an enlargement of adipocytes by excessive fat storage. With an increasing prevalence, childhood obesity is correlated with endothelial dysfunction, inflammation and oxidative stress conducting to the development of other diseases not only in children but also during adulthood. In other hand, numerous children practice exercise of high duration or intensity in high competition sports, which can have harmful effects at physical, physiological and psychological level. In high competition young athletes, oxidative stress and immunosuppression can happen leading to an elevated risk of infection. However, an improved lipid profile is found in childhood athletes. Thus, the objective of the present study was to analyze the impact of childhood obesity as well as intense swimming training in body composition, inflammation and lipid profile, through blood analysis, bioimpedance and immunodetection of the pro-inflammatory cytokines (IL-6 and TWEAK), a myokine (Myostatin) and an acute-phase protein (CRP). For that, 24 young people were recruited into three groups: obese, athlete and lean. The obese group had high levels of body fat, an atypical lipid profile (low HDL and high LDL), high levels of lactate dehydrogenase in the blood indicating tissue damage, chronic inflammation (high IL-6, CRP and TWEAK) and low muscle mass (high Myostatin) without muscle damage (low CK). However, low serum levels of hepatic enzyme (AST and ALT) in these obese children do not associate obesity with liver disease. In other hand, intense physical exercise is not a harmfull activity for young athletes, since the lipid profile is improved and the increased levels of inflammatory markers is not significant. The main benefit of intensive training is the decreased levels of glucose being a protective role for diabetes.

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List of abbreviations

ActRII	Activin type 2 receptor
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
ADMA	Asymmetric dimethylarginine
AGE	Advanced glycosylation end product
Akt	Protein kinase B
ALT	Alanine transaminase
AMPK	Activated protein kinase
AP-1	Activator protein-1
AST	Aspartate transaminase
ATM	Adipose tissue macrophage
ATP	Adenosine triphosphate
AU	Arbitrary units
BCA	Bicinchoninic acid
BH4	Tetrahydrobiopterin
BMI	Body mass index
CCR2	C-C motif chemokine receptor 2
CCR5	C-C motif chemokine receptor 5
Chol	Cholesterol
CK	Creatine kinase
CRP	C-reactive protein
EDTA	Ethylenediamine tetraacetic acid
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal–regulated kinase
ET-1	Endothelin 1
$FADH_2$	Flavin adenine dinucleotide
FFA	Free fatty acid
FFM	Free fat mass
FM	Fat mass
Fn14	Fibroblast growth factor-inducible 14
FVIIa	Coagulation factor VIIa
Hb	Hemoglobin
HDL	High-density lipoprotein
HDL_C	High-density lipoprotein cholesterol
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IKB	Inhibitor of NF-kB
IKK	IKB kinase
IL-10	Interleukin 10
IL-1ra	Interleukin 1 receptor antagonist
IL-1β	Interleukin 1 beta

IL-6	Interleukin-6
Ins-R	Insulin receptor
IR	Insulin resistance
IRF	Interferon regulatory factor
IRS-1	Insulin receptor substrate 1
JAK	Janus kinase
JNK	Jun N-terminal kinase
K^+	Potassium
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDLc	Low-density lipoprotein cholesterol
LepRb	Leptin receptor
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCP-1	Monocyte chemoattractant protein-1
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MPV	Mean platelet volume
mTOR	Mammalian target-of-rapamycin
NADH	Nicotinamide adenine dinucleotide
NF-kB	Nuclear factor k light-chain-enhancer of activated B cells
NIK	NF-κB-inducing kinase
NLRP3	NLR Family, Pyrin Domain Containing 3
NO	Nitric oxide
NOS	Nitric oxyde synthase
oxLDL	Oxidized low-density lipoprotein
P	Phosphore
PAR1	Protease activated receptor 1
PAR2	Protease activated receptor 2
PBS	Phosphate-buffered saline
PDW	Platelet distribution width
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PPARα	Peroxisome proliferator-activated receptor alpha
RAS	Renin-angiotensin system
RBC	Red blood cell
RDW	Red cell distribution width
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOCS-3	Suppressor of cytokine signaling 3
STAT	Signal transducer and activator of transcription
TBK1	TANK binding kinase 1
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline and Tween-20
TCA	Tricarboxylic acid
TChol	Total cholesterol

TF	Tissue factor
TG	Triglycerides
TGF-β	Transforming growth factor-beta
Th_1	Type 1 T helper cells
Th_2	Type 2 T helper cells
TLR	Toll-like receptor
TNF-R	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
TRAF6	TNF receptor associated factor 6
Treg	Regulatory T cell
TWEAK	Tumor necrosis factor weak inducer of apoptosis
UPR	Unfolded protein response
WAT	White adipose tissue
WBC	White blood cell
WHO	World Health Organization

1. Introduction

1.1.Childhood obesity and its epidemiology

Obesity results from an excess of adipose tissue which may impair health [1]. In children, obesity is defined by a body mass index (BMI) above the 95th percentile for age and gender while in overweight the BMI is between the 85th and the 95th percentiles [2]. The World Health Organization (WHO) describes childhood obesity as "one of the most serious public health challenges of the 21st century" since childhood obesity comes with numerous life-threatening co-morbidities [3]. Respiratory disorders, hypertension, hyperglycemia, hyperlipidemia, insulin and leptin resistance and impaired cardiac function are some of the most notorious implications of childhood obesity which lead to consequences in both childhood and adulthood and reduce the life span [1,4,5].

In the past three decades the prevalence of obesity and overweight has risen substantially [6]. Since 1980 obesity has more than doubled worldwide. In 2013 more than 42 million children under the age of five were overweight [3]. In Portugal there were 28.7% obese boys and 27.1% obese girls in the year of 2013. The main reason for this is the fact that the energy intake is much higher than the energy expenditure due to lifestyle behaviors [6]. Overweight and obesity, as well as their related co-morbidities, are largely preventable with interventions in diet and/or physical activity which may be implemented in schools, at home and in the society [3,7,8].

The identification of obesity biomarkers related with endothelial dysfunction, inflammation and oxidative stress are crucial to identify early triggers for cardiovascular disease and to prevent other co-morbidities [9].

1.2. Childhood obesity and its systemic impact

Obesity can be caused by a low resting metabolic rate, a poorly developed satiety response, environmental factors and/or family behavior and stress or anxiety [10].

Regardless of the cause, obese people have a compulsive behavior related to food intake due to the complex interactions that exist between the brain, that controls food intake and satiety, and the adipose tissue, which regulates relative fat mass. The mesoaccumbens dopamine system is essential to sustain appropriate control over feeding and associated behaviors. However, consumption of highly palatable energy-dense food deeply impacts this system and downstream signaling cascades in cortical and limbic regions [11]. Furthermore, obesity is characterized by a decreased population of dopamine D2 receptors in the striatum contributing to compulsive food intake and consequent weight gain [12].

White adipose tissue (WAT) is the most relevant depot in obesity [13]. It is not only a site of storage of excess energy from food intake, leading to the expansion of adipose tissue due to the increase in the number of adipocytes (hyperplasia) or an enlargement in the size of adipocytes (hypertrophy), but also an endocrine organ secreting multiple adipokines (chemokines, cytokines and hormones) [14]. Adipokines play crucial roles in the regulation of appetite and satiety control, energy expenditure, insulin sensitivity and secretion, endothelial function and blood pressure [15]. The adipokines leptin and adiponectin are primarily secreted by adipocytes playing a major role in the pathogenesis of obesity and its comorbidities [15]. While leptin suppresses appetite and increases energy expenditure via the hypothalamus [16], adiponectin plays a role in lipid and energy metabolism being anti-inflammatory, anti-atherogenic and insulin sensitizing, determining the endothelial function and suppressing the generation of ROS (reactive oxygen species) in lean individuals [17]. Contrary to adiponectin, leptin levels are increased in obese individuals due to the enhanced body fat percentage. Nevertheless, obese individuals do not have a decreased appetite because they do not respond to leptin in an adequate manner [18,19]. On the other hand, obesity involves a state of chronic low-grade systemic inflammation [15]. CRP (C-reactive protein) is an acute-phase reactant protein synthesized mainly in the liver and its production is induced by pro-inflammatory cytokines like IL-6 (interleukin-6) and TNF-α (tumor necrosis factor alpha). CRP is an inflammatory biomarker and it is capable of modulating endothelial function, being increased in adipose tissue and serum of obese individuals [18,20].

Moreover, the enlargement of adipocytes by fat storage induces adipose tissue hypoxia, and the secretion of high levels of inflammatory cytokines is associated with adipocyte dysfunction, particularly mitochondrial stress and disrupted endoplasmic

reticulum function [1]. Activation of inflammatory cascades is initiated by the secretion of chemo-attractants, such as MCP-1 (monocyte chemoattractant protein 1), which attract immune cells into the tissue (e.g., M1 polarized macrophages, pro-inflammatory phenotype, that secrets the cytokines IL-6, TNF-α and IL-1β) **(Figure 1)** [13,21]. Lymphocytes also infiltrate human adipose tissue being CD_4^+T cells along with CD_8^+T cells the majority of T-lymphocytes [13,15] Examples of dysregulated CD_4^+T cells are the Th_1 (type 1 T helper cells) which are increased in obesity and Th_2 (type 2 T helper cells) and Treg (regulatory T cell), both diminished [22,23]. The decrease in the Treg cells in obesity may promote the infiltration of macrophages in adipose tissue and, thereby, increase the production of inflammatory cytokines. CD_4^+T lymphocytes in adipose tissue are involved in the regulation of body weight, adipocyte hypertrophy and glucose tolerance [24]. CD_8^+T cells are increased and they are involved in the initiation and propagation of inflammatory cascades in obese adipose tissue and have major roles in macrophage differentiation, activation and migration [25]. However, eosinophils are diminished in obesity since they have been suggested to play a protective role. [26].

This chronic inflammation is deeply involved in insulin resistance (IR) [15]. Insulin is a hormone that acts on skeletal muscle, adipose tissue and liver controlling glucose homeostasis and in the production of the potent vasodilator nitric oxide (NO) from vascular endothelium through the PI3K/Akt (phosphoinositide 3-kinase/protein kinase B) pathway. Insulin also stimulates the production of the vasoconstrictor ET-1 (endothelin 1) through MAPK (mitogen-activated protein kinase) pathway [27]. However, in obesity elevated pro-inflammatory cytokines such as TNF-α and IL-6 secreted by adipose tissue macrophages via autocrine/paracrine signaling might block the insulin action [19]. Due to this, vasodilation is compromised decreasing the glucose uptake in target tissues **(Figure 1)** [27].

Inflammation related to obesity and the consequently increase of ROS and RNS (reactive nitrogen species) are potential triggers of early endothelial dysfunction in obese children and its early detection is a major clinical goal [9]. Endothelial dysfunction is an early stage of atherosclerosis and it may start in obese children. It describes the functional alteration of the endothelium largely due to the impaired bioavailability of NO, a marker of arterial damage [28,29]. Cytokines and cellular adhesion molecules are produced by dysfunctional endothelium and the macrophages and leukocytes recruited lead to the

formation of atherosclerotic plaques [30]. Endothelial function is affected by leptin, IL-6 and CRP through direct or indirect mechanisms such as reduction of NO production and stimulation of inflammation-oxidative stress pathways **(Figure 1)** [9]. Obesity-related reduced endothelial function has also been associated with hypoadiponectinaemia. The lack of NO production induces vasoconstriction, leucocyte adherence, platelet activation and oxidative stress. All this leads to endothelial dysfunction in obese children [9].

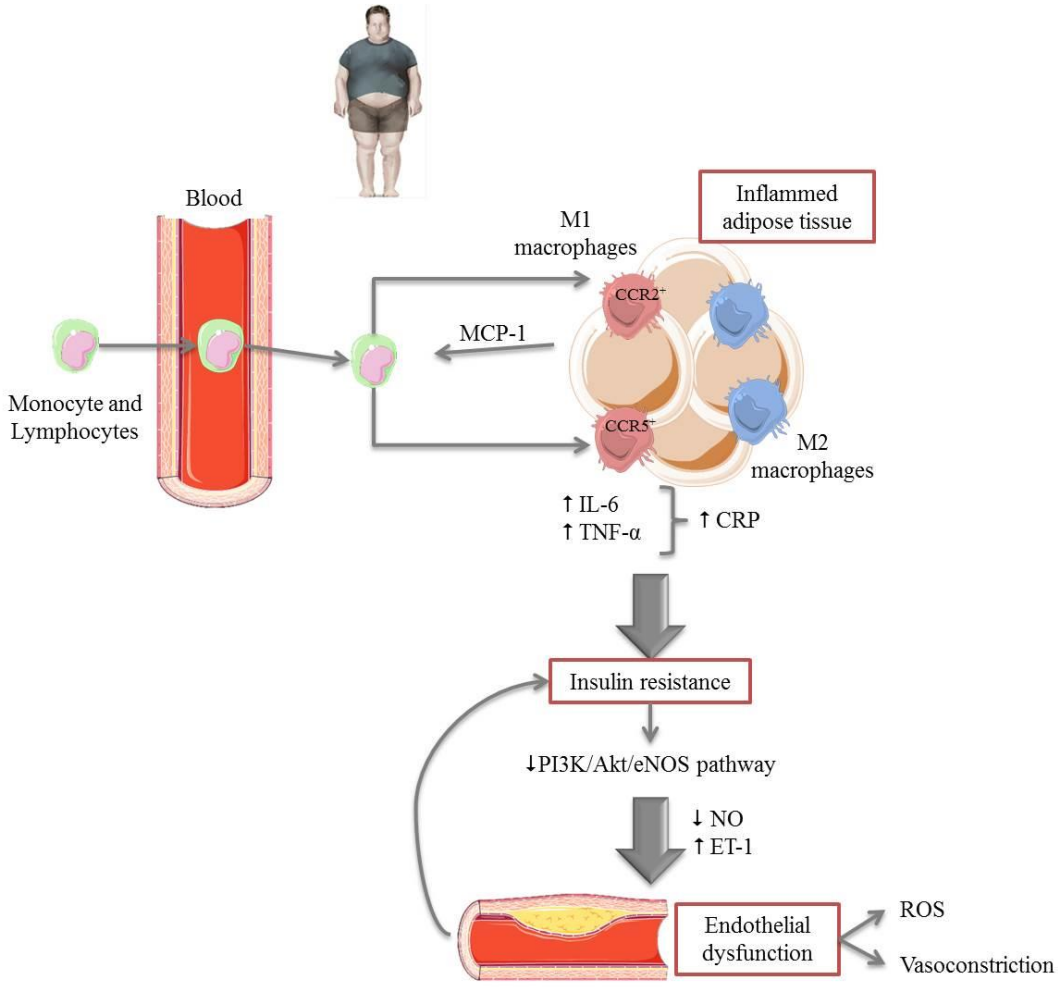

Figure 1 – Activation of inflammatory cascades in childhood obesity leading to the development of adipose tissue inflammation and insulin resistance and consequently contributing for endothelial dysfunction (figure was produced using Servier Medical Art) [9,15,27,29]. **Akt:** Protein kinase B, **CCR2$^+$:** C-C motif chemokine receptor 2, **CCR5$^+$:** C-C motif chemokine receptor 5, **CRP**: C-reactive protein, **eNOS:** Endothelial Nitric Oxide Synthase, **ET-1:** Endothelin 1, **IL-6:** Interleukin-6, **MCP-1:** Monocyte chemoattractant protein-1, **NO:** Nitric oxide, **PI3K:** Phosphoinositide 3-kinase, **ROS:** Reactive oxygen species, **TNF-α:** Tumor necrosis factor alpha.

Obesity affects other peripheral tissues involved in glucose homeostasis (liver and skeletal muscle) inducing morphological and metabolic alterations because adipose tissue dysfunction is accompanied by lipid spillover in the circulation and accumulation of the lipids in non-adipose tissues [13,31]. Increased pro-inflammatory cytokines secreted by WAT and the consequent immune cell infiltration cause synergistic effects on hepatic and skeletal metabolism related to insulin resistance, increasing lipid storage in the liver and decreasing glucose transport in the skeletal muscle [13,32].

The serum profile of obese children is mainly characterized by elevated circulating levels of triglycerides (TG) (≥110 mg/dl) and low levels of high-density lipoprotein (HDL) (≤40 mg/dl), increased inflammatory biomarkers like CRP, IL-6, leptin, fibrinogen, e-selectin and intercellular adhesion molecule-1 (ICAM-1) and decreased levels of other markers, such as adiponectin [9,33,34]. Increased oxidative stress markers like malondialdehyde (MDA), F2-isoprostanes and advanced oxidation protein products are also present, while there are low antioxidant defenses (e.g., catalase and superoxide dismutase) [9].

1.3. Childhood obesity and its cellular impact

Adipocytes and immune cells are activated by adipose tissue signals like lipids, cytokines or lipopolysaccharides (LPS) through the TNFR (tumor necrosis factor receptor) and the TLR (Toll-like receptor) pathways. NF-kB (nuclear factor k light-chain-enhancer of activated B cells) and MAPK pathways are activated by those events, resulting in the translocation of transcription factors to the nucleus, transcriptional activation, and pro-inflammatory cytokine production [13]. NF-kB pathway is activated through the activation of IKK (IKB kinase) complexes and degradation of IKB (inhibitor of NF-kB) that allows NF-kB binding to inflammatory gene promoters in the nucleus. MAPK cascade phosphorylate JNK (Jun N-terminal kinase) which activates AP-1 (activator protein-1) [35].

IRFs (interferon regulatory factor) are phosphorylated in the TRAF6/TBK1 (TNF receptor associated factor 6/TANK binding kinase 1) cascade after activation of TLRs by adipose-derived signals, it dimerizes with STAT (signal transducer and activator of transcription) in the JAK/STAT cascade and, in the end, IFN-stimulated genes promoters are expressed [21].

Insulin resistance in the adipocytes can be induced by all the above inflammatory signals [13,21]. Increases in TNF-α, CRP and IL-6 decrease NO production by decreasing eNOS (endothelial nitric oxide synthase) expression and by activating serine kinases through JNK pathway which phosphorylate IRS-1 (Insulin receptor substrate 1), inactivating it **(Figure 2)**. This leads to the inhibition of PI3K/Akt/eNOS pathway. Moreover, TNF-α and CRP enhance the production of vasoconstrictor ET-1 [27]. So, in obesity vasodilation is compromised due to insulin resistance contributing to endothelial dysfunction. However, endothelial dysfunction per se can contribute to insulin resistance as represented in **Figure 1**, since reduced microvascular vasodilation in skeletal muscle and decreases the delivery of insulin and glucose to skeletal muscle [13,19]. Endothelial dysfunction happens due to an imbalance between relaxing and contractile endothelial factors (NO and ET-1) playing a central role in the pathogenesis of cardiometabolic diseases [36]. The production of NO in lean individuals happen when PI3K/Akt pathway is activated leading to phosphorylation of eNOS which, with the cofactor BH4 (tetrahydrobiopterin), converts L-arginine to L-citrulline and NO. However, in obese children, shear stress and insulin resistance characterize endothelial dysfunction leading to the decrease of NO production. The eNOS activation is inhibited by oxidized LDL (low-density lipoprotein) and ADMA (asymmetric dimethylarginine) and eNOS and BH4 do not bind due to BH4 poor availability. The consequence of this is the increased generation of ROS which leads to reduced bioavailability of NO and vasoconstriction [19].

In adipocytes, suppression of Akt happens by a mechanism that requires the FVIIa (coagulation factor VIIa) and the TF (tissue factor) cytoplasmic domain. TF is linked to a procoagulant state in obesity being a risk factor for the development of arterial thrombosis [37]. TF acts thought G protein-coupled protease activated receptors (PAR2, PAR1) since TF–PAR2 signaling in adipocytes contributes to diet-induced obesity by decreasing metabolism and energy expenditure. TF-FVIIa-PAR2 signaling is affected by several hormonal and metabolic changes contributing to elevated TF expression in obesity. This leads to increased insulin resistance and decreases adiponectin synthesis by adipocytes which inhibits AMPK (activated protein kinase), PPARα (peroxisome proliferator-activated receptor alpha) and β-oxidation in a β-arrestin recruitment dependent manner [38]. TF–FVIIa–PAR2 signaling activates and sustains M1 polarization of ATMs (adipose tissue macrophages), contributing to insulin resistance **(Figure 2)** [37].

CCR5 (C-C motif chemokine receptor 5) promotes obesity-induced inflammation and insulin resistance as represented in **Figure 1** [39]. First, MCP-1 and other chemokines, ligands for CCR5, are secreted by adipocytes in obesity. Due to this, monocytes exit the bone marrow in a CCR2 and/or CCR5-dependent manner being recruited to inflamed tissues and activated as M1/M2 ATMs. $CCR2^{+}$ and/or $CCR5^{+}$ macrophages accumulate and maintain the inflammation as M1 macrophages in obese adipose tissue producing cytokines [15,39].

Leptin resistance is the inability of obese individuals to respond to elevated levels of leptin. This can happen due to a decrease in leptin transport from the blood to the hypothalamus, a critical site for leptin actions. Central leptin signaling is involved in leptin resistance too. The leptin receptor (LepR) activates JAK/STAT so modulating PI3K/Akt and ERK (extracellular signal–regulated kinases) signaling pathways. However, leptin capacity to activate STAT3 and PI3K is decreased in obese individuals since SOCS-3 (suppressor of cytokine signaling 3) is a negative regulator of leptin receptor signaling, and possibly it is elevated in obesity [40]. The main effects of leptin in obesity include increased phosphorylation of eNOS, the induction of ROS and the increased expression of inflammatory markers (TNF-α and IL-6) **(Figure 2)** [19].

Contrary to leptin, adiponectin concentrations are lower in obesity being negatively correlated with BMI. Adiponectin through two transmembrane receptors (AdipoR1 and AdipoR2) activates specific pathways (AMPK, PPAR-a, and p38 MAPK) promoting an increase of fatty acid oxidation and glucose uptake in both liver and skeletal muscle, and a decrease of hepatic gluconeogenesis [18]. Decreased adiponectin levels and its function in obesity make people more susceptible to atherosclerosis because this protein is no longer able to protect against vascular injury and atherogenesis. It also leads to an increase in free fatty acids and consequently a decrease in glucose uptake. Due to this the risk of developing insulin resistance and cardiovascular disease is higher [41].

TNF-α is a pro-inflammatory cytokine enhanced in obesity, secreted by monocytes and macrophages, via the activation of MAPK and NF-kB signaling pathways. TWEAK (tumor necrosis factor weak inducer of apoptosis), a cytokine of the TNF superfamily, is gaining attention as an important player in chronic inflammatory diseases [42]. It is an important mediator of skeletal muscle wasting and metabolic dysfunction and, in the heart, TWEAK can induce cardiac remodelling [43,44]. It acts through Fn14 receptor being

produced by a variety of cell types including macrophages, skeletal muscle, and adipocytes [45]. The inflammatory effects of TWEAK in subcutaneous and visceral adipocytes are mediated through the activation of both canonical and non-canonical NF-kB pathway and by the induction of the MAPKs, ERK1/2 and p38 **(Figure 2)** [42].

Increased levels of TWEAK and its receptor Fn14 have been observed in adipose tissue of obese individuals [45]. This results in the release of other inflammatory cytokines like IL-6, an interleukin secreted by WAT, skeletal muscle, and the liver acting via the JAK/STAT and MAPK pathways **(Figure 2)**. The expression of IL-6 in WAT and plasma is correlated with BMI and up-regulated by insulin and TNF-α (13). IL-6 induces the release of hepatic CRP and this acute-phase protein suppresses adiponectin production through PI3K/Akt pathway, playing a major role in metabolism and insulin sensitivity [46]. CRP is not only an inflammatory marker but also modulates endothelial function since induces the expression of MCP-1 via increased secretion of ET-1 and IL-6. In the other hand, CRP decreases NO activity. Due to these factors, excessive body fat can contribute to cardiovascular diseases since adipokines play a major role in endothelial dysfunction [47].

Other protein increased in obese individuals is the Myostatin, a potent inhibitor of skeletal muscle growth member of the transforming growth factor-β (TGF-β) superfamily. It is a secreted muscle factor with the criteria of myokine [48,49]. Myostatin is not only restricted to skeletal muscle; it is also detected in other tissues like adipose tissue and in heart muscle [50]. Muscle growth inhibition can be produced by Myostatin through Smad signaling and MAPK signaling pathways. However, this protein plays an important role in metabolism too by mammalian target-of-rapamycin (mTOR) inhibition **(Figure 2)** [48,51]. Myostatin overexpression reduces muscle mass, fiber size and myonuclei number. Interestingly, aerobic and strength training reduce skeletal muscle myostatin expression associated to increased muscle strength, hypertrophy and metabolic homeostasis. However, the increased Myostatin expression in muscle is also associated with metabolic disorders, such as obesity [49,51]. Higher serum Myostatin levels are also associated with overweight and obesity [52].

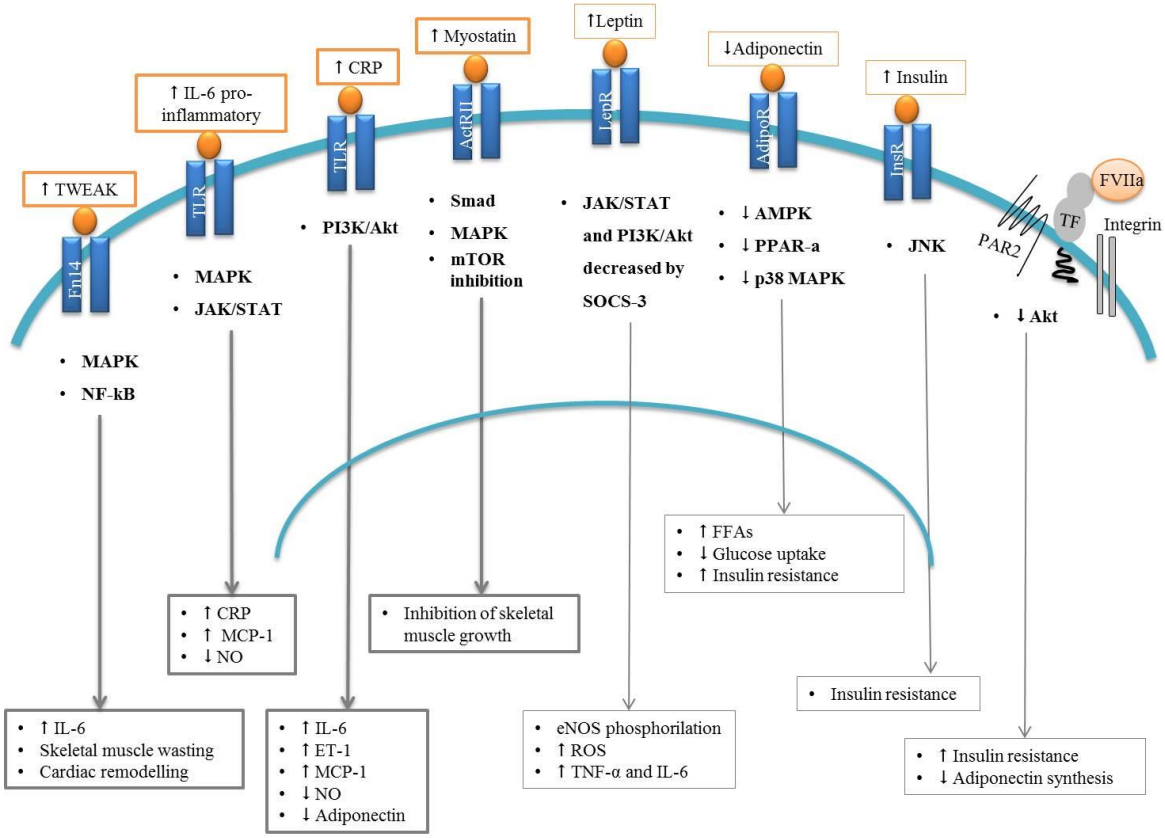

Figure 2 – Obesity signaling pathways of inflammation and respective outputs. **ActRII:** Activin type 2 receptor, **Akt:** Protein kinase B, **AMPK:** Activated protein kinase, **AP1:** Activator protein-1, **ATMS:** Adipose tissue macrophages, **ERK:** Extracellular signal–regulated kinase, **FVIIa:** Factor VIIa, **IkB:** Inhibitor of NF-kB, **IKK:** IKB kinase, **IL-6:** Interleukin-6, **Ins-R:** Insulin receptor, **IRS:** Insulin receptor substrate, **JAK:** Janus kinase, **JNK:** Jun N-terminal kinase, **MAPK:** Mitogen-activated protein kinase, **mTOR:** Mammalian target-of-rapamycin, **NF-kB:** Nuclear factor k light-chain-enhancer of activated B cells, **NIK:** NF-κB-inducing kinase, **P:** Phosphore, **p38:** Protein 38, **PAR2:** Protease activated receptor 2, **PI3K:** Phosphoinositide 3-kinase, **PPARα:** Peroxisome proliferator-activated receptor alpha, **ROS:** Reactive oxygen species, **SMAD: SOCS-3:** Suppressor of cytokine signaling 3, **STATs:** Signal transducer and activator of transcription, **TF:** Tissue factor, **TLRs:** Toll-like receptors, **TNF-R:** Tumor necrosis factor receptor, **TNF-α:** Tumor necrosis factor alpha, **TWEAK:** Tumor necrosis factor weak inducer of apoptosis. **Orange**: proteins studied in this work.

Childhood obesity is strictly related to redox unbalance. Low concentrations of ROS and RNS are crucial for the normal cell redox status, cell function and intracellular signaling. However, in obesity ROS and RNS are over-produced damaging DNA, proteins, carbohydrates and lipid constituents and thus compromising cell function [53]. Oxidative stress is not only a consequence but also a trigger for obesity. A high fat diet can lead to oxidative stress through different mechanisms: altered lipid (hyperlipidemia) and glucose

metabolism (hyperglycemia), chronic inflammation, tissue dysfunction, hyperleptinemia and altered expression of miRNAs. On the other hand, oxidative stress increases adipocyte proliferation, differentiation and size and alters food intake by acting on hypothalamic neurons that control satiety [54].

The typical hyperlipidemia of obesity consists of increased FFA (free faty acids), triglycerides and LDL_C (low density lipoprotein cholesterol) and decreased HDL_C (high density lipoprotein cholesterol) with HDL dysfunction [55]. When it happens, adipocytes became unable to function as a storage organ and lipotoxicity occurs. This leads to mitochondrial uncoupling, due the accumulation of ATP (adenosine triphosphate), promoting free radical release. Other consequence of hyperlipidemia is ER (endoplasmic reticulum) stress that activates UPR (unfolded protein response). If UPR is prolonged, ROS production is caused by the persistent oxidative protein folding machinery [54].

Obesity is a major cause of type 2 diabetes, clinically known as hyperglycemia, which results in altered glucose homeostasis caused by faulty signal transduction via the insulin signaling proteins [56]. Hyperglycemia leads to the overproduction of NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide), through the glycolytic pathway and the TCA (tricarboxylic acid) cycle [54]. As a result of this, the voltage gradient across the mitochondrial membrane increases reaching a threshold that blocks the electron transfer inside complex III. Due to this, electrons back up to coenzyme Q which donates the electrons one at a time to molecular oxygen generating superoxide [57]. Superoxide leads to the activation of polyol pathway, hexosamine pathway, AGEs (advanced glycosylation end products) and PKC (protein kinase C), alternative pathways which induce enhancing ROS and RNS production or impairing antioxidant defenses [54].
Elevated plasma leptin levels or hyperleptinemia have been correlated with hyperphagia and insulin resistance which are obesity markers. Hyperleptinemia can induce oxidative stress, because increases the oxidation of mitochondria and peroxisomal fatty acids [54,58].

Other mechanism for the increased oxidative stress in obese individuals is the stimulation of the renin-angiotensin system (RAS) by tissue dysfunction. RAS regulates blood pressure, fluid and electrolyte balance and is implicated in the pathogenesis of obesity, inflammation, oxidative stress and insulin resistance. Adipose tissue secretes angiotensin II, generated in RAS from its precursor angiotensinogen. Angiotensin induces

ROS in adipocytes, which increases the release of peroxidation products from macrophages. ROS induction inhibits insulin signaling which reduces glucose uptake leading to IR [59].

A vicious circle happens related to oxidative stress in obesity because there are pro-inflammatory transcription factors like NF-κB and AP-1 that are redox sensitive but, in the other hand, inflammatory cytokines enhance ROS production [54].

1.4. Exercise training – a double-edged sword?

Physical inactivity is a major risk for chronic diseases and is becoming a major public health problem. In other hand, physical activity and regular exercise training results in numerous beneficial adaptations and decreases the risk of several chronic diseases including cardiovascular diseases and events that are common to obesity [48]. Some of the beneficial effects of exercise training are attributable to an increase in energy expenditure and consequently a reduction in the accumulation of fat mass [60]. Regular exercise can reduce abdominal adiposity and improve weight control; enhance lipoprotein profiles, glucose homeostasis and insulin sensitivity; reduce blood pressure and systemic inflammation; improve cardiac function and endothelial function [61]. Physical activity has also been associated with skeletal muscle modifications thus enhancing endurance and metabolic efficiency and psychological benefits in young people [3,62].

Any kind of exercise training has an acute response and chronic adaptations which cannot be viewed separately. Physical exercise can be considered as a biological stress. Muscle contractions disturb the body intracellularly causing homeostatic responses such as altered blood flow and increased heart rate, breathing rate, oxygen consumption and body temperature. However, these physiological, metabolic, and neuromuscular changes return to baseline levels after exercise. Frequent repetitions of the exercise leads to chronic adaptations involving tissue remodeling and/or altered regulation of the central nervous system leading to a muscle more resistant to fatigue and stronger [63]. **Table 1** provides a summary of the some of the main acute and chronicle adaptations to physical exercise.

Table 1 – Physiological responses to acute endurance exercise and adaptations of human organs and tissues to repeated sessions of aerobic training [61,64].

Organ/Tissue	Acute response	Chronic adaptations
Brain	↑ Blood distribution	↑ Psychological well-being ↓ Anxiety and depression ↑ Expression of neurotrophic factors ↑ Memory and cognitive function ↑ Quality of sleep and Neurogenesis
Lung	↑ Breathing rate ↑ Ventilation and gas exchange ↑ Blood flow and distribution	↑ Blood flow and gas exchange reserve ↑ Volumes ↑ Capillary surface area ↑ Cardio respiratory function
Heart	↑ Heart rate ↑ Cardiac output ↑ Oxygen consumption ↑ Blood flow distribution	↑ Cavity size ↑ Wall thicknesses ↓ Blood flow and oxygen consumption
Skeletal muscle	↑ Metabolism and blood flow ↑ Oxygen extraction and consumption ↑ Mechanical strains ↑ IL-6 anti-inflammatory ↑ATP (rate of sweating)	↓ Resting and exercising blood flow ↑ Max. blood flow ↑ Mitochondriogenesis ↑ Fatty acid oxidation ↓ TWEAK and Myostatin
Liver, pancreas, gut and kidneys	↓ Blood flow ↑ Metabolism ↑ CRP	↓ CRP
Bone marrow	↑ Blood flow ↑ Mechanical strain ↑ Release of steam cells	↑ Eosinophils and M2 macrophages
Adipose tissue	↑ Metabolism ↑ IL-6 pro-inflammatory	↓ Abdominal adiposity ↑ Weight control ↑ Beige adipocytes ↓ IL-6 pro-inflammatory
Vasculature	↑ Arterial dilatation ↑ Capillary pressure and energy substrate exchange ↑ Blood distribution ↑ Venoconstriction	↑ Arterial diameters ↑ Capillary density ↑ Endothelial function
Blood	↑ Hemoconcentration ↑ Oxygen content ↑ Levels of energy substrates	↑ Blood volume ↑ Red blood cells and HDL ↓ Hemoglobin, LDL, triglycerides ↓ Blood pressure and coagulation
Glands	↑ Rate of sweating ↑ Body temperature ↑ Secretion of stress hormones (cortisol)	↑ Sweating capacity

Aerobic exercise, like swimming or the exercise practiced in school, is the type of exercise that results in cardiovascular changes [65]. Chronic adaptations to aerobic exercise have hemodynamic, morphologic and metabolic results. The hemodynamic consequences include a decrease in the resting heart rate and blood pressure, an increase in work capacity and maximal oxygen consumption, and a faster recovery from an exercise bout. The morphologic changes like an increase in myocardial mass and in coronary artery size make heart to function better under stress [66].

While a single bout of aerobic exercise increases pro-inflammatory and oxidative markers and leads to antioxidants depletion, long-term exercise, known as regular training

or chronic exercise, protects against systemic inflammation and oxidative stress, because it can reduce plasma levels of CRP, IL-6 and F2-isoprostane, and improve lipid and glycemic profiles and endogenous antioxidants [65]. Anti-inflammatory cytokines such as IL-10 and IL-1ra (interleukin 1 receptor antagonist) are increased and exercise training enhances the levels of eosinophils, M2 alternatively activated macrophages and $CD_4^+CD_{25}^+$ Treg lymphocytes (**Figure 4**) [60].

Since long-term exercise has anti-inflammatory action, pro-inflammatory cytokines like IL-6 and TWEAK as well as the myokine Myostatin and the acute-phase protein CRP decrease with this type of exercise (**Figure 4**). IL-6 is an interleukin relevant in exercise. It has pro- and anti-inflammatory, as well as metabolic effects [65]. Adipose tissue contributes to the production of TNF, which is reflected by elevated levels of pro-inflammatory IL-6 and C-reactive protein. However, during exercise, anti-inflammatory IL-6 is produced by muscle fibers through a TNF-independent pathway (glycogen/MAPK activation pathway rather than through NF-κB) stimulating the appearance in the circulation of other anti-inflammatory cytokines like IL-1ra and IL-10 (**Figure 3**) [67,68]. Muscle release of IL-6 increases with exercise intensity and duration, additionally long-term exercise training reduces resting levels of pro-inflammatory markers such as CRP and IL-6 (**Figure 4**) [48,65]. Exercise training preventes the activation of TWEAK/NF-kB pathway which improves cardiac, skeletal muscle and metabolic functions (**Figure 3**) [44]. Aerobic training has the capacity to reduce the risk of cardiovascular disease development since decreases serum CRP concentration in healthy humans [69].

The principal anti-inflammatory effect of exercise may be mediated through effects on TLR pathway activation. The availability of endogenous TLR4 ligands (FFAs and LDLs) as well as the expression of TLR4 and it activation signaling is decreased due to exercise training. This is associated with reduced activation of JNK and decreased serine phosphorylation of IRS-1, activating insulin signaling pathway and consequently increasing glucose uptake [60]. Regular exercise leads to greater insulin-stimulated IRS-1-associated PI3K activation in human skeletal muscle. TLR4 also plays a key role in pro-inflammatory NF-kB pathway activation being decreased by chronic exercise (**Figure 3**) [70]. NF-kB dependent cytokine releases activate the JNK/MAPK pathway which results in the serine phosphorylation of IRS-1 and the phosphorylation of AP-1. The phosphorylation of serine residues in IRS-1 leads to an impairment in the ability of IRS-1

to activate downstream PI3K-dependent pathways which may cause insulin resistance [70]. However, exercise is capable of decrease this pathways activation leading to insulin signaling and attenuation of inflammatory signaling as well as inflammasome activation reduction [60].

Similar with TLRs, ligands for the NLRP3 (NLR family, pyrin domain containing 3) inflammasome like FFAs and oxidized LDL are decreased by exercise, reducing the activation of the NLRP3 inflammasome and, therefore, reducing the levels of IL-1β (**Figure 3**) [60].

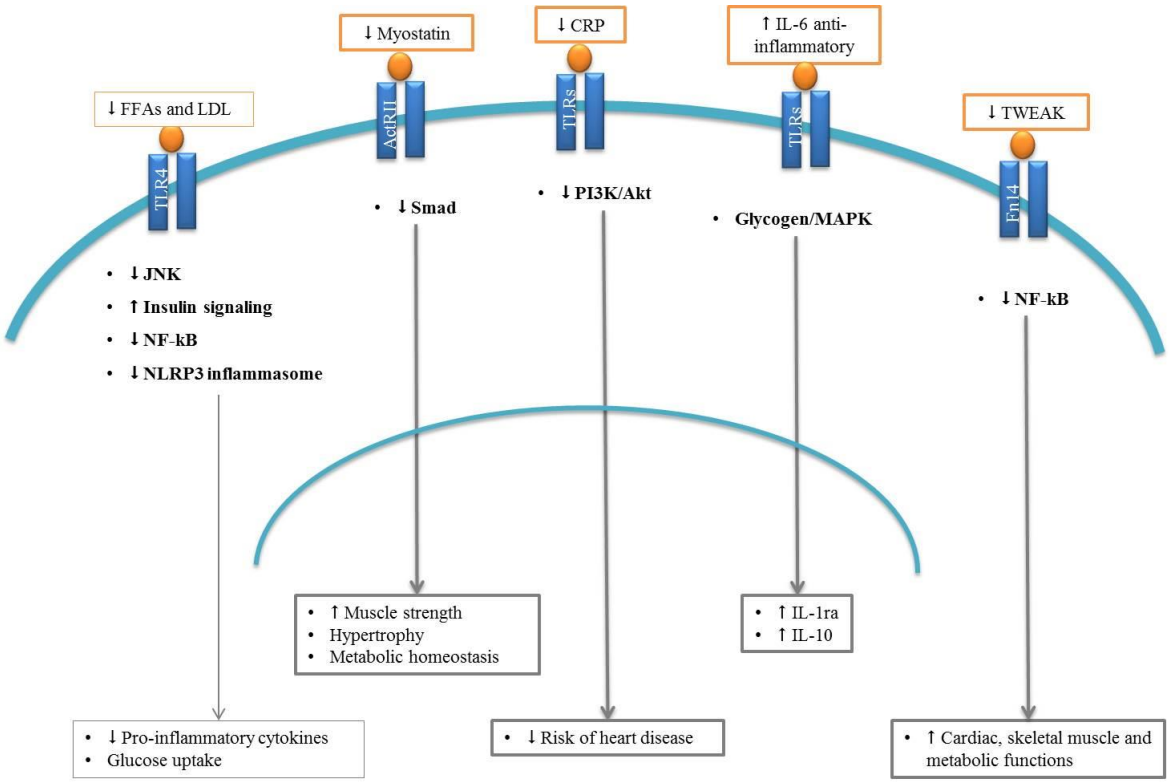

Figure 3 – Exercise signaling pathways of inflammation and respective outputs. **AP1:** Activator protein-1, **ASC:** adaptor protein ASC**, FFAs:** Free fatty acids, **IKB:** Inhibitor of NF-kB, **IKK:** IKB kinase, **IL-1β:** Interleukin 1 beta, **IRS-1:** Insulin receptor substrate 1, **JNK:** Jun N-terminal kinase , **K^+:** Potassium, **MAPK:** Mitogen-activated protein kinase, **NF-kB:** Nuclear factor k light-chain-enhancer of activated B cells, **NIK:** NF-κB-inducing kinase, **NLRP3:** NLR Family, Pyrin Domain Containing 3, **oxLDL:** Oxidized low-density lipoprotein, **P:** Phosphor, **PI3k:** Phosphoinositide 3-kinase, **ROS:** Reactive oxygen species, **TLRs:** Toll-like receptors. **Orange**: proteins studied in this work.

Aerobic exercise has impact in the regulation of adipose tissue mass and function. With chronic exercise the size of adipocytes are reduced, enhancing fat mobilization being small adipocytes associated with a protective role [71]. A sedentary lifestyle contributes to

an adipose tissue formed by white adipocytes which store fat and contain large lipid droplets contributing to obesity. High deposition of visceral adipose tissue, a hormonally active component of total body fat, is associated with medical disorders being more dangerous than subcoutaneous adipose tissue [72]. However, in exercise-trained individuals, adipose tissue adopts characteristics of beige adipocytes (multilocular cells with lipid droplets, increased vascularization and mitochondrial enrichment). This leads to a concomitant increase in glucose uptake in oxidative skeletal muscle and brown adipose tissue since brown adipocytes appear under conditions of increased energy expenditure, such as cold and exercise [73,74].

The production of ROS increases during prolonged inactivity and during exercise. Prolonged periods of skeletal muscle inactivity lead to muscle atrophy, a loss of muscle protein and strength. Oxidative stress plays a big role in this problem regulating proteolytic pathways. Oxidative stress during prolonged skeletal muscle inactivity may be due to ROS production by xanthine oxidase pathway, NADPH oxidase, production of NO via nitric oxide synthase and production of superoxide radicals by mitochondria [75]. However, ROS generated during exercise have a physiological role in the adaptation to exercise leading to the development of antioxidant defenses such as superoxide dismutase, peroxidases and glutathione. Cell has become well equipped to deal with the normal production of ROS during an acute bout of exercise and due to this, regular exercise is considered an antioxidant [76]. This paradox is explained by the hormesis theory which says that chemicals and toxic substances that are deleterious at high doses can have a low-dose beneficial effect. The beneficial dose of ROS occurs after exercise adaptation and involves the redox regulation of NF-kB and the activation of MAPK, PI3K/Akt or p53 pathways [48].

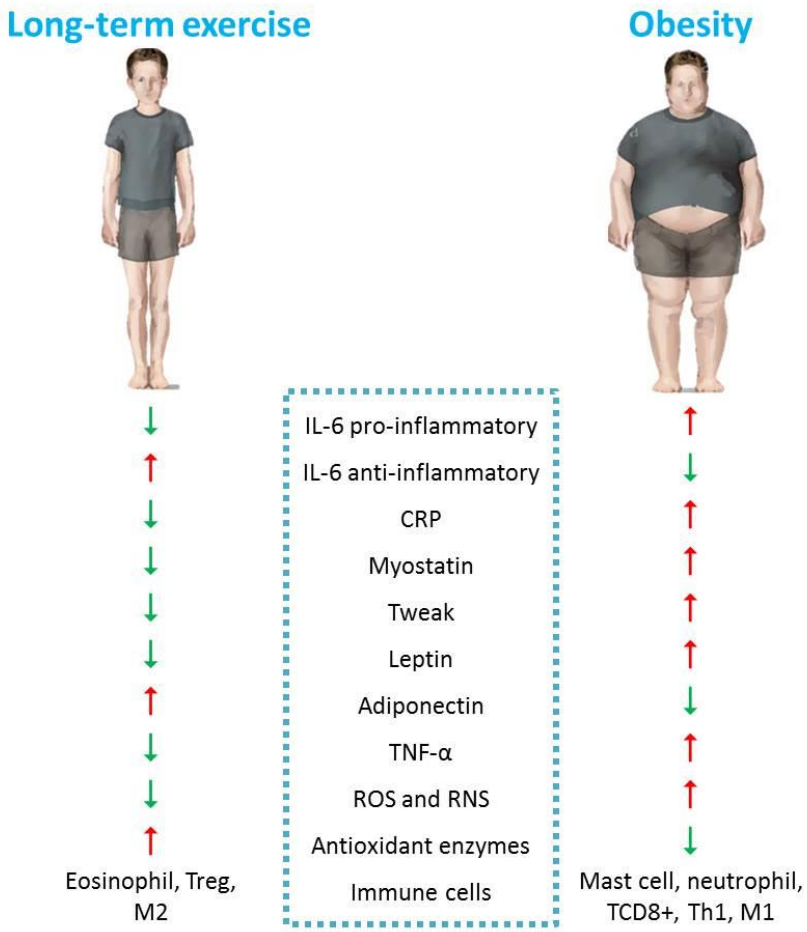

Figure 4 – Molecular comparison between long-term exercise [77,78] and obesity [1,9,25,34] in children (figure was produced using Servier Medical Art). **CRP:** C-reactive protein, **IL-6:** Interleukin 6, **M1:** Macrophages type 1, **M2:** Macrophages type 2, **RNS:** Reactive nitrogen species, **ROS:** Reactive oxygen species, **TCD8$^+$:** CD8$^+$ T cells, **Th1:** Type 1 T helper cells, **TNF-α:** Tumor necrosis factor alpha, **Treg:** Regulatory T cell, **TWEAK:** Tumor necrosis factor weak inducer of apoptosis.

As previously said, regular exercise has been accepted as a cardiovascular protector but is important to define the most beneficial mode, duration, intensity and frequency of the exercise [66]. Some controversy exists regarding the health benefits of intense exercise, like high competition sports. Swimming is one of the most exciting sports due to the tournament competitive schedules and the severe training programs. Training can improve the performance of the swimmers but excessive training can also cause negative health sequelae such as muscle damage and airway epithelial damage due to the intensive training combined with exposure to chlorination's products [79,80].

Intensive training in young athletes can predispose these children to certain health problems including cardiac, nutrition, maturation, musculoskeletal, and psychological difficulties [81,82]. Compared with non-athletes, child athletes have superior cardiac functional capacity. Bradycardia, cardiomegaly, heart murmurs, and ECG changes are typically observed in adult endurance athletes as well as cardiac injury or dysfunction. Consequently, a significant elevation of the serum concentration of troponin I, the sensitive and specific cardiac biomarker, can be observed [83]. Among young athletes it is not frequent. However, in young swimmers can be observed lower resting heart rates and echocardiographic manifestations of chronic left ventricular volume overload [84].

Physical activity stimulates positive responses in musculoskeletal structures if the stress is repetitive and the athlete train below the threshold for injury. However, excessive sports training in child and adult athletes can lead to tissue breakdown and overuse injuries (tendinitis, apophysitis, stress fractures) predisposing the child and adolescent to repetitive stress. Therefore, training starting before and maintained throughout puberty can alter growth rates. Young athletes require an adequate diet, because nutritional needs are increased by both training and the growth process. Athletes often avoid foods containing iron and calcium, because of its fat content. However, adequate iron stores are important to provide adequate oxygen transport, muscle aerobic metabolism, and cognitive function and sufficient dietary calcium is essential for a normal bone growth and prevention and healing of stress fractures [84].

Intensive sports training might delay sexual maturation, so athletic girls tends to experience menarche at a later age than nonathletic ones due to a lower body fat, training, stress or undernutrition. Secondary amenorrhea can happen too being attributable to an imbalance between energy expenditure and caloric intake [82]. Child athletes may be more at risk for heat-related injuries in hot, humid conditions, because they sweat less, create more heat per body mass and acclimatize slower to warm environments than adults [84].

Psychological difficulties can also be present in young athletes being at risk of emotional and social problems. Burnout, anxiety, depression and attrition are increased in early athletes mainly due to an excessive coach, parent, and athlete pressure [81,82].

In highly trained adult athletes more physical complications can happen like alterations in systemic immune parameters revealing suppressed immunity during and immediately after training sessions. The mechanism of this immunosuppression is

unknown, but may be mediated by hormonal changes associated with exhausting exercise. Some changes are transitory being related with the acute effect of intense exercise. However, changes in leucocyte numbers and the capacity of immune cells (monocytes, dendritic cells and neutrophils) to produce inflammatory cytokines in response to an external stimulation is affected [85,86]. Due to this the response to pathogens could be compromised contributing to an elevated risk of infection [87,88].

Intensive physical exercise causes an increase in the levels of WBC (white blood cells) activation products like ROS and proteases, whose damaging vascular endothelium and accelerate RBCs (red blood cells) aging or its premature removal. ROS may oxidatively modify LDL, which when oxidized is more avidly taken by macrophages than native LDL. Consequently, circulating monocytes are recruited leading to the accumulation of resident macrophages and accelerated uptake by them and, finally, fatty streak is formed. Thus, oxidative stress, proteolytic stress and high levels of Chol (cholesterol) and LDL_C could lead to the development of premature atherosclerotic lesions in high competition athletes [89].

In summary, it is needed a reevaluation of current views in high competition sports requirements. A threshold may be found in the intensity, duration and frequency of physical exercise that, whenever it is exceeded, could lead to illness [83,89]. Furthermore, more studies have to be performed to fully understand the signaling pathways which are affected by chronic intensive exercise.

2. Aims

Childhood obesity is considered a complex and multifactorial disease increasing the risk of mortality during adulthood. Its origins can be genetic, behavioral, socioeconomic or environmental with an increasing worldwide prevalence. Obesity is associated with chronic systemic inflammation with activation of innate immune system in the adipose tissue. This promotes an increase of the production and release of pro-inflammatory cytokines that trigger the systemic acute-phase response [90].

Physical inactivity is a public health problem and it is a major risk for childhood obesity. Thereby, regular exercise training is important to improve health not only in obese children but in all population [48]. In other hand, nowadays more and more children practice intensive training which can predispose the childhood athletes to several health problems [79].

Due to the factors mentioned above, the main goal of this thesis was:

To analyze the impact of childhood obesity as well as intense swimming training in body composition, inflammation and lipid profile, through blood analysis, bioimpedance and immunodetection of the pro-inflammatory cytokines (IL-6 and TWEAK), a myokine (Myostatin) and an acute-phase protein (CRP) in the serum of obese and athlete children.

3. Methods

3.1. Participants

Twenty-four children aged between 13 and 19 years old – eight in each group (obese, lean and athletes) – were recruited for this study. The inclusion criterion for obese children was having a body mass index higher than 95th percentile for their age while for not overweight children the BMI was between the percentile 5th and 85th. The third group of children included not overweight athletes (swimmers from local swim club) whose inclusion criterion was having five or more weekly training sessions. The above criteria were present during evaluation and in the 12 months preceding the study. Children with medical contraindication for performing exercise, diagnosed pathology or with musculoskeletal injury that prevents the implementation of evaluations were excluded from the study.

This study was performed according to the recommendations of the Helsinki Declaration for human investigations. The aim of the study as well as it nature, benefits and risks was explained to participants, parents and teachers or trainers. Parents gave their written consent and the study protocol was approved by the ethics committee of sports college of University of Porto (CEFADE.2014).

3.2. Study design

The present cross sectional study comprised physical evaluations and blood collection for further analysis. All participants were subjected to the same protocol (**Figure 5**).

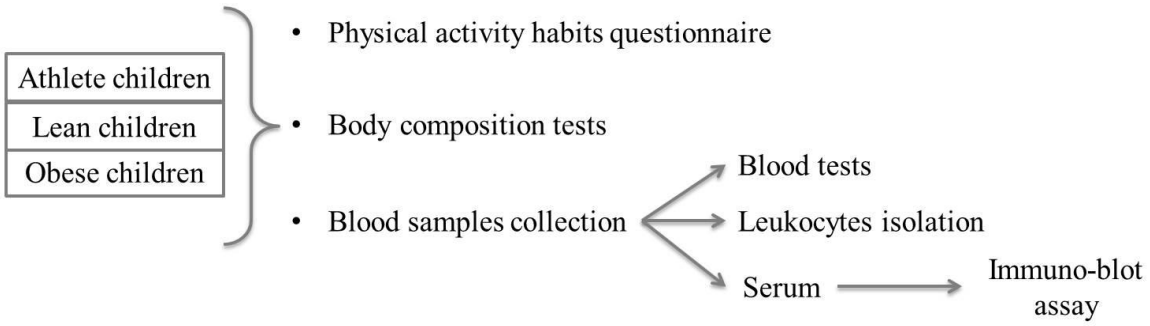

Figure 5 – Study design schematization.

3.3. Physical activity and sports participation

The physical activity and the sports participation of the participants were evaluated through a questionnaire which concerns physical activity habits [91]. The questionnaire originally consists of twenty-three questions, however, for the study's purposes, only six questions were included in the analyses (**Appendix 8.1**).

3.4. Evaluation of body composition

Body mass and height were measured following standard procedures. Body height was measured to the nearest mm in bare or stocking feet with participants standing upright against a stadiometer (Seca 285, Seca, Birmingham, United Kingdom). Weight was measured with participants lightly dressed, using a medical Body Composition Analyzer (seca mBCA 525, Birmingham, United Kingdom). Body mass index (BMI) was calculated from the ratio of body weight (kg)/body height (m^2). A bioimpedance analysis was performed with the medical Body Composition Analyzer (seca mBCA 525, Birmingham, United Kingdom) and provided data regarding fat mass (FM), fat-free mass (FFM) and appendicular skeleton muscle mass.

3.5. Blood samples collection

Blood tests were performed to the non-overweight, athlete and obese children in an automated analyser in a clinical laboratory.

Venous blood was collected for assessment of biochemical parameters. The blood was processed in order to obtain serum and leukocytes. Serum was obtained from blood through centrifugation at 1200 g for 15 minutes and stored in 500 μL aliquots at -80ºC. Leukocytes isolation from anticoagulated blood (EDTA) was performed using Histopaque protocol (see below).

3.6. Preparation of protein extracts from leukocytes

Histopaque kit (Sigma-Aldrich Histopaque-1077) was used to isolate leukocytes from whole blood according to the manufactures' instructions. Briefly Histopaque is a sterile, endotoxin tested solution of polysucrose and sodium diatrizoate. During centrifugation, erythrocytes are aggregated by polysucrose and rapidly sediment (pellet formation); granulocytes become hypertonic, which increases their sedimentation rate, resulting in the formation of a pellet in the centrifuge tube bottom; mononuclear cells remain at the plasma/Histopaque interface. Blood collected in EDTA (ethylenediamine tetraacetic acid) (2 mL) was carefully added onto the Histopaque (2 mL) in a conical centrifuge tube. The tubes were centrifuged at 400 g for exactly 30 minutes at room temperature and after centrifugation the upper layer was aspirated by a Pasteur pipete and discarded. The opaque interface was transferred into a clean conical centrifuge tube and cells were washed by adding 6,6 mL of PBS (phosphate-buffered saline). Samples were centrifuged at 250 g for 10 minutes and after that the supernatant was aspirated and discarded. Cell pellet was ressuspended in 3,3 mL of PBS and gently mixed and was then centrifuged at 250 g for 10 minutes. After, the cell pellet was ressuspended in 0,5 mL of PBS and stored at -20ºC.

Isolated leukocytes were lysed. First, samples were unfreezed, centrifuged at 126 g for 10 minutes and the supernantant removed. After, 30 μL of 1 % SDS was added to each sample pellet and the sample was placed on ice for 20 minutes after which they were centrifuged for 8 minutes at 1300 g and the supernatant was then sonicated and finally saved. The protein content was measured using the bicinchoninic acid (BCA) protein assay (Pierce). The samples were stored at -80ºC for future use.

3.7. Immuno-blot assay

To perform the slot-blot, the 24 serum samples were 20x diluted with 1x PBS. The same volume of sample (100 μL) was applied into the membrane. The membranes were incubated in 10% methanol for activation, washed in water and posteriorly in 1x TBS. Then each sample was applied into the membrane and slot-blotted under vacum (BioRad Portugal, Sintra, Portugal) for a few seconds until all the sample was transferred to the nitrocellulose membrane.

Ponceau staining was performed to confirm the presence of protein in each sample (protein bands in all the membranes incubated with samples) and also to be used as the loading control. For Ponceau preparation 0,1 g of Ponceau S was dissolved in 5 mL of acetic acid and filled up to 100 mL of dd H_2O. After the transfer, the membrane was washed in 1x Tris Buffered Saline and Tween-20 (TBS-T) and then incubated with Ponceau on a shaker for 5 minutes. The membrane was then washed with distilled water until the protein bands were well defined. To remove the staining of the membrane, it was destained completely by repeated washing in distilled water.

Nitrocellulose membranes were blocked for 1 hour in 1x TBS-T/5 % low fat milk at room temperature with slow shaking. Next, the appropriate primary antibody was added and incubated for 1 hour (**Table 2**). All antibodies were diluted 1:1000 in 1x TBS-T/5 % low fat milk. Then, the membranes were washed three times with 1x TBS-T for 15 minutes each. The membrane was then incubated with the anti-rabbit infrared secondary antibody (1:5000, LI-COR Biosciences UK Ltd, Cambridge, UK) for 1 hour in the dark. Then, the membranes were washed two times, one with 1x TBS-T for 15 minutes and the other one with 1x TBS during 15 minutes. The detection was performed in the Odyssey Infrared Imaging System (LI-COR Biosciences UK Ltd).

Table 2 – Primary antibodies used.

Antibody	Dilution	Antibody	Suplier
Myostatin	1:1000	Rabbit polyclonal anti-GDF8 (ab996)	Abcam
TWEAK	1:1000	Rabbit polyclonal anti-TWEAK (ab37170)	Abcam
CRP	1:1000	Rabbit monoclonal anti-CRP (ab32412)	Abcam
IL-6	1:1000	Rabbit polyclonal anti-IL6 (ab6672)	Abcam

3.8. Statistical analysis

An observational study approach was used to assess the impact of obesity and practice of intensive extra school sport in the health of young people.

Initially was conducted an exploratory data analysis (EDA) using graphical techniques (bar charts, box and scatter plots) and quantitative analysis (statistical measures and frequency tables) in order to characterize each group, detect possible extreme outliers and measurement error. To analyze the differences among group means, we performed the ANOVA or the Kruskal Wallis test. Following in order to identify the alterations between groups, were conducted tests of the equality of means for independent samples: t-test or Mann-Whitney test. The assumptions of the tests were performed (Shapiro Wilk test and box-plot). In each group, the correlations between the main measures were tested with the Pearson correlation coefficent. The significance level was set at 0.05. Statistical analysis was conducted using IBM SPSS Statistics Software 22.

4. Results

4.1. Characterization of study groups

In order to evaluate de impact of obesity and intensive physical exercise in the health of young people, 24 children aged between 13 and 19 years old were recruited, being 79% of them boys. These children were divided into three groups: the lean group (control group) constituted by 8 young boys who only practice school sport and have a BMI within the 5^{th} and 85^{th} percentiles, the athlete group with 8 young boys who practice intensive extra school sport and have a BMI between 5^{th} and 85^{th} percentiles and the obese group formed by 8 children (boys and girls) who only practice school sport and have a BMI higher than the 95^{th} percentile **(Table 3)**.

Table 3 – Descriptive statistics of age and gender of the lean, athlete and obese children and its respective sport type.

	Lean group	**Athlete group**	**Obese group**
Age (years)	15 ± 1	14 ± 1	17 ± 1
Gender	8 male	8 male	5 female, 3 male
Sport	School sport (1h40min/week)	Intensive extra school sport – swimming (6h/week)	School sport (1h40min/week)

Age is presented as Mean values ± Standard Deviation.

The hemogram and biochemical analysis of the participants' blood samples, the body composition' results and the levels of protein obtained through immuno-blot were submitted to a statistical analysis. The results with statistical significance are presented in the following sections. In the **Appendix 8.3,** the remaining data can be found with descriptive statistics.

4.2. Body composition parameters in lean, athlete and obese children

The participants' body composition is described in **Table 4**. To confirm if there was a significant difference in body composition parameters between the three groups of children, the ANOVA was performed. We observed a significant difference between groups in body weight ($F_{2,20}$ = 18.706, p<0.001), BMI ($F_{2,20}$ = 29.028, p<0.001), FM (kg) ($F_{2,19}$ = 36.477, p<0.001) and FM (%) ($F_{2,19}$ = 51.972, p<0.001). Weight, BMI and FM (kg and %) were significantly high in the obese group in comparison to the lean group and also in relation to the athlete group. FFM (kg) (lean: 51.5±7.4, athletes: 53.7±10.3, obese: 48.2±6.6; p>0.05) was not significantly differente between groups.

The differences between lean/athlete, lean/obese and athlete/obese groups could be consulted in **Appendix 8.3.1**.

Table 4 – Results of the body composition parameters in lean, athlete and obese children. Two independent samples were compared (lean/athlete, lean/obese and athlete/obese).

Variable	Lean group	Athlete group	Obese group	Study Groups		N	p-value (2-tailed)
Weight (kg)	61.8±12.9	52.3±8.7	86.2±12.4	Lean	Athlete	7	0.120[a)]
					Obese	7	0.002[a)] *
				Athlete	Obese	8	0.000[a)] *
BMI (kg/m^2)	22.3±4.3	19.8±2.0	31.5±3.2	Lean	Athlete	7	0.160[a)]
					Obese	7	0.000[a)] *
				Athlete	Obese	8	0.000[a)] *
FM (%)	15.7±6.7	11.0±7.0	37.9±6.1	Lean	Athlete	7	0.220
					Obese	7	0.000 [a)] *
				Athlete	Obese	7	0.000 [a)] *
FM (kg)	10.3±6.0	6.1±3.9	32.5±6.0	Lean	Athlete	7	0.150[a)]
					Obese	7	0.000[a)] *
				Athlete	Obese	7	0.000[a)] *

[a)] – Independent sample t-test. Equal variances assumed (Levene´s test for equality of variables – p-value>0.05);
* - The difference is significant at the 0.05 level;
Data are presented as Mean values ± Standard Deviation;
BMI: body mass index, **FM**: fat mass.

4.3. Hemogram parameters in lean and athlete children

A hemogram was performed with the blood samples from both lean and athlete groups. A descriptive statistics of the results was obtained and is described in **Table 5**. To confirm if there was a significant difference in the hemogram variables between the two groups with available data for this variable, t-Student Test and Mann-Whitney test were performed for independent samples. Lean and athlete children have similar results in these blood parameters, being all of them within the reference values, with the exception of MCHC which was significantly lower in the athlete group.

The differences between lean/athlete groups could be consulted in **Appendix 8.3.2**.

Table 5 – Results of the hemogram parameters in lean and athlete children. Two independent samples were compared (lean/athlete).

Variable	Lean group	Athlete group	Reference Values	Study Groups		N	p-value (2-tailed)
Erythrocytes (10^12/L)	5.06±0.52	5.09±0.27	4.50-5.50	Lean	Athlete	8	0.890[a)]
Hb (g/dL)	14.61±1.41	14.19±1.57	13.00-17.00	Lean	Athlete	8	0.800[c)]
Hematocrit (%)	42.23±3.89	42.36±4.14	40.00-50.00	Lean	Athlete	8	0.960[c)]
MCV (fL)	83.48±2.42	83.50±10.00	83.00-101.00	Lean	Athlete	8	0.200[c)]
MCH (pg)	28.89±0.67	27.99±3.74	27.00-34.00	Lean	Athlete	8	0.960[c)]
MCHC (g/dL)	34.60±0.61	33.46±0.86	31.50-34.50	Lean	Athlete	8	0.009[a)] *
RDW (%)	13.25±0.47	13.24±1.01	1.50-14.50	Lean	Athlete	8	0.280[c)]
Leukocytes (10^9/L)	6.65±2.13	6.45±0.88	4.00-11.00	Lean	Athlete	8	0.810[a)]
Neutrophils (%)	43.24±3.92	47.58±4.79	40.00-80.00	Lean	Athlete	8	0.070[a)]
Eosinophils (%)	8.05±6.19	3.39±1.41	1.00-7.00	Lean	Athlete	8	0.070[b)]
Basophils (%)	0.40±0.18	1.45±3.14	0.00-3.00	Lean	Athlete	8	0.960[c)]
Lymphocytes (%)	41.24±6.47	40.84±4.65	20.00-50.00	Lean	Athlete	8	0.890[a)]
Monocytes (%)	7.08±1.10	7.88±1.26	2.00-10.00	Lean	Athlete	8	0.200[a)]
Platelets (10^9/L)	231.50±39.61	232.13±35.67	150.00-400.00	Lean	Athlete	8	0.970[a)]
MPV (fL)	10.15±0.65	10.73±0.89	7.40-11.00	Lean	Athlete	8	0.160[a)]
PDW (fL)	12.00±1.25	12.56±1.43	9.80-16.20	Lean	Athlete	8	0.420[a)]

[a)] – Independent sample t-test. Equal variances assumed (Levene´s test for equality of variables – p-value>0.05);

[b)] – Independent sample t-test. Equal variances not assumed (Levene´s test for equality of variables – p-value<0.05);

[c)] – Mann-Whitney test.

* - The difference is significant at the 0.05 level;

Data are presented as Mean values ± Standard Deviation;

Hb: haemoglobin, **MCH**: mean corpuscular hemoglobin, **MCHC**: mean corpuscular hemoglobin concentration, **MCV**: mean corpuscular volume, **MPV**: mean platelet volume, **PDW**: platelet distribution width, **RDW**: red cell distribution width.

4.4. Biochemistry parameters in lean, athlete and obese children

The biochemistry parameters of the three groups of children were obtained through blood sample analyses. A descriptive statistics of the results can be observed in the **Table 6**. To confirm if there was a significant difference in the biochemistry parameters between lean, athlete and obese groups, ANOVA was performed for parametric parameters. We observed a significant difference between groups in glucose ($F_{2,21}$ = 5.082, p=0.016), HDL ($F_{2,21}$ = 49.979, p<0.001), LDL ($F_{2,21}$ = 26.571, p<0.001), LDH ($F_{2,21}$ = 6.133, p=0.008) and CK ($F_{2,21}$ = 26.293, p<0.001). Glucose was statistically lower in the athlete group in comparison to the lean group; HDL and CK were statistically lower in the obese group in comparison to both the lean and the athlete groups; LDL and LDH were statistically high in the obese group in comparison to the lean group and the athlete group. The Kruskal Wallis Test was performed for the non-parametric parameters of biochemistry in order to determine if there was a significant difference between the three groups of children. We observed a significant difference between groups in AST (H = 14.140, p=0.001). Mann-Whitney test for independent samples was performed for the biochemistry parameters with statistical significance in Kruskal Wallis Test. AST was statistically lower in the obese group in comparison to both lean and athlete groups.

TChol (mg/dL) (lean: 137±22, athlete: 138±29, obese: 162±21; p>0.05), TG (mg/dL) (lean: 61±27, athlete: 57±34, obese: 75±23; p>0.05), ALT (U/L) (lean: 21±8, athlete: 20±5, obese: 19±5; p>0.05) and Albumin (g/dL) (lean: 5.1±0.7, athlete: 4.7±0.1, obese: 5±0; p>0.05) were not significantly different between group.

The main results are within the reference values for the three groups of children except HDL, LDL and LDH in the obese group.

The differences between lean/athlete, lean/obese and athlete/obese groups could be consulted in **Appendix 8.3.3**.

Table 6 – Results of the biochemistry parameters in lean, athlete and obese children. Two independent samples were compared (lean/athlete, lean/obese and athlete/obese).

Variable	Lean group	Athlete group	Obese group	Reference values	Study Groups		N	p-value (2-tailed)
Glucose (mg/dL)	88.0±6.0	75.0±6.0	85.0±12.0	70.0-110.0	Lean	Athlete	8	0.000[b] *
						Obese	8	0.490[a]
					Athlete	Obese	8	0.060[a]
HDL (mg/dL)	43.0±10.0	50.0±13.0	7.0±0.0	>45.0	Lean	Athlete	8	0.250[a]
						Obese	8	0.000[b] *
					Athlete	Obese	8	0.000[b] *
LDL (mg/dL)	81.0±18.0	77.0±21.0	141.0±20.0	<130.0	Lean	Athlete	8	0.680[a]
						Obese	8	0.000[a] *
					Athlete	Obese	8	0.000[a] *
AST (U/L)	27.0±5.0	31.0±4.0	20.0±4.0	<50.0	Lean	Athlete	8	0.080[c]
						Obese	8	0.001[c] *
					Athlete	Obese	8	0.001[c] *
LDH (U/L)	200.0±18.0	203.0±25.0	304.0±113.0	<248.0	Lean	Athlete	8	0.810[a]
						Obese	8	0.020[b] *
					Athlete	Obese	8	0.030[b] *
CK (U/L)	161.0±62.0	222.0±50.0	51.0±19.0	<171.0	Lean	Athlete	8	0.052[a]
						Obese	8	0.000[b] *
					Athlete	Obese	8	0.000[b] *

[a] – Independent sample t-test. Equal variances assumed (Levene´s test for equality of variables – p-value>0.05);

[b] – Independent sample t-test. Equal variances not assumed (Levene´s test for equality of variables – p-value<0.05);

[c] – Mann-Whitney test.

* - The difference is significant at the 0.05 level;

Data are presented as Mean values ± Standard Deviation;

ALT: alanine transaminase, **CK**: creatine kinase, **HDL**: high density lipoprotein, **LDH**: lactate dehydrogenase, **LDL**: low density lipoprotein.

4.5. Levels of IL-6, CRP, Myostatin and TWEAK in lean, athlete and obese children

The levels of IL-6, CRP, Myostatin and TWEAK were evaluated in the serum samples of the lean, athlete and obese children through immuno-blot assay (**Appendix 8.2.**). A descriptive statistic of the results was obtained and bar charts were performed for each variable in order to visualize the variability and the dispersion of the data (**Figure 6**). The ANOVA was performed in order to test if there was a significant difference in protein levels between the three groups. We observed a significant difference between groups in IL-6 ($F_{2,21}$ = 10.000, p=0.001), CRP ($F_{2,21}$ = 23.013, p<0.001), Myostatin ($F_{2,21}$ = 8.645, p=0.002) and TWEAK ($F_{2,21}$ = 5.682, p=0.011). IL-6, CRP and Myostatin were statisticaly high in the obese group in comparison to both the lean (p=0.001; p<0.001; p=0.005) and the athlete (p=0.006; p<0.001; p=0.022) groups. TWEAK was statistically high in the obese group in comparison to the lean group (p=0.001).

The differences between lean/athlete, lean/obese and athlete/obese groups could be consulted in **Appendix 8.3.4**.

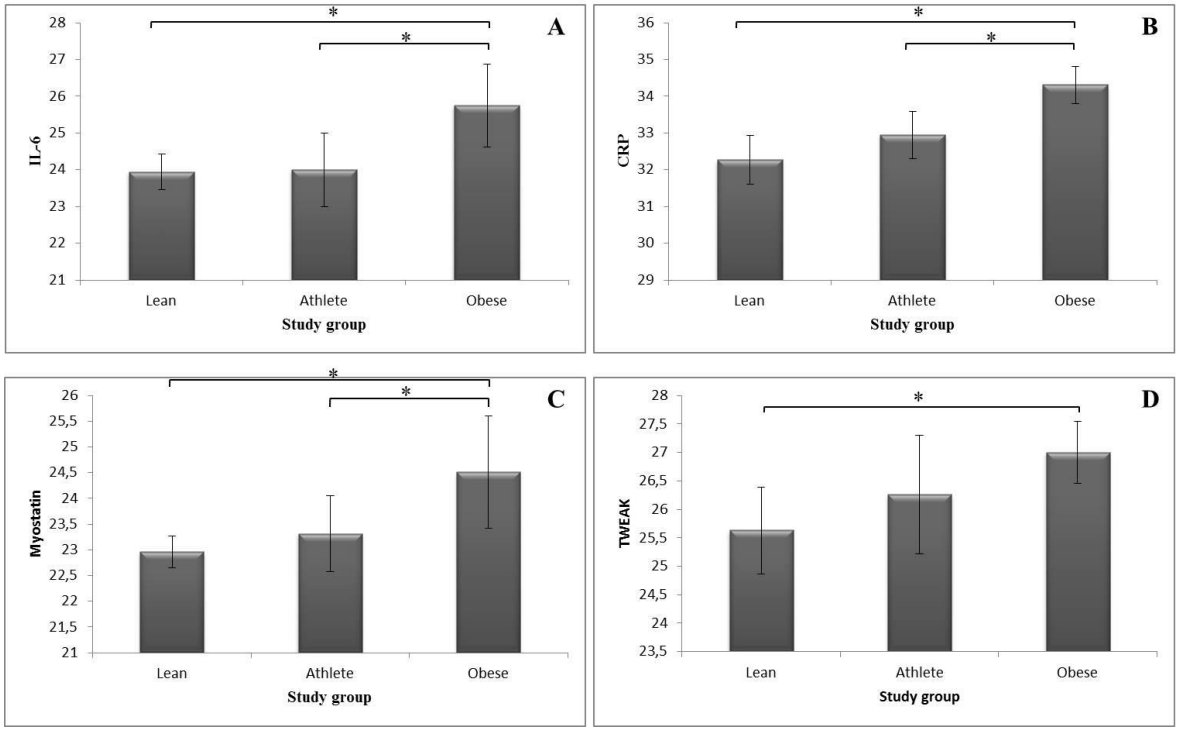

Figure 6 – Results of the serum parameters evaluated in the lean, athlete and obese groups [**(A)** IL-6 (AU), **(B)** CRP (AU), **(C)** Myostatin (AU) and **(D)** TWEAK (AU)].

4.6. Correlations in the lean, athlete and obese children

Body composition, the blood biochemical variables, and the levels of IL-6, CRP, Myostatin and TWEAK were tested for possible correlations between them. These correlations were tested by performing a Pearson correlation and the results with statistical significance are presented in the following sections.

4.6.1. Correlations between body composition and biochemistry parameters

Regarding to the relationship between body composition parameters and biochemistry parameters in the lean group, a moderate positive linear correlation was found between BMI and CK (r=0.794), while a strong positive linear correlation was found between weight and CK (r=0.806) and between FFM and CK (r=0.805) (**Table 7**).

Table 7 – Pearson Correlation Coefficient (r) with p-value between the body composition and the biochemistry parameters in the lean group.

LEAN GROUP		Glucose (mg/dL)	TChol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)	AST (U/L)	ALT (U/L)	LDH (U/L)	CK (U/L)	Albumin (g/dL)
Height (cm)	**r**	-,120	.201	.355	.072	-.063	.156	.440	-.637	.123	.347
	Sig.	.798	.666	.435	.877	.894	.739	.323	.124	.792	.445
	N	7	7	7	7	7	7	7	7	7	7
Weight (kg)	**r**	-.525	-.247	-.517	-.181	.586	-.112	.229	.111	.806*	.157
	Sig.	.226	.593	.234	.698	.167	.811	.622	.813	.029	.738
	N	l7	7	7	7	7	7	7	7	7	7
BMI (kg/m2)	**r**	-.529	-.345	-.684	-.230	.654	-.171	.081	.380	.794*	.037
	Sig.	.222	.449	.090	.620	.111	.714	.863	.400	.033	.938
	N	7	7	7	7	7	7	7	7	7	7
FM (%)	**r**	-.693	.076	-.402	.140	.706	.065	.412	.187	.637	.378
	Sig.	.084	.871	.371	.765	.076	.889	.358	.688	.124	.403
	N	7	7	7	7	7	7	7	7	7	7
FM (kg)	**r**	-.584	-.086	-.465	-.029	.682	-.050	.342	.051	.750	.272
	Sig.	.169	.854	.293	.950	.092	.915	.453	.914	.052	.555
	N	7	7	7	7	7	7	7	7	7	7
FFM (kg)	**r**	-.444	-.364	-.530	-.294	.474	-.159	.121	.148	.805*	.051
	Sig.	.318	.422	.221	.523	.283	.733	.796	.751	.029	.914
	N	7	7	7	7	7	7	7	7	7	7

* - Correlation is significant at the 0.05 level (2-tailed).
ALT: alanine transaminase, **AST:** aspartate transaminase, **BMI**: body mass index, **CK**: creatine kinase, **FM**: fat mass, **FFM**: free fat mass, **HDL**: high-density lipoprotein, **LDH**: lactate dehydrogenase, **LDL**: low-density lipoprotein, **TChol**: total cholesterol, **TG**: triglycerides.
Blue: lean group's correlations.

In the athlete group, a strong negative linear correlation was obtained between height and LDH (r=-0.849), weight and TChol (r=-0.824) and FFM and LDH (r=-0.868). In the other hand a moderate negative linear correlation was found between weight with LDL (r=-0.767) and LDH (r=-0.723) while a moderate positive linear correlation was obtained between weight and albumin (r=0.762) (**Table 8**).

Table 8 – Pearson Correlation Coefficient (r) with p-value between the body composition and the biochemistry parameters in the athlete group.

ATHLETE GROUP		Glucose (mg/dL)	TChol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)	AST (U/L)	ALT (U/L)	LDH (U/L)	CK (U/L)	Albumin (g/dL)
Height (cm)	**r**	.633	-.673	-.443	-.680	.049	-.583	-.140	-.849**	-.069	.654
	Sig.	.092	.067	.272	.064	.908	.129	.740	.008	.871	.079
	N	8	8	8	8	8	8	8	8	8	8
Weight (kg)	**r**	.488	-.824*	-.667	-.767*	.104	-.680	-.385	-.723*	-.225	.762*
	Sig.	.220	.012	.071	.026	.806	.064	.346	.043	.592	.028
	N	8	8	8	8	8	8	8	8	8	8
BMI (kg/m2)	**r**	.089	-.590	-.621	-.477	.133	-.441	-.505	-.193	-.295	.523
	Sig.	.834	.123	.100	.233	.754	.274	.202	.647	.478	.184
	N	8	8	8	8	8	8	8	8	8	8
FM (%)	**r**	.084	-.107	-.170	.030	-.136	-.496	-.345	.289	-.496	.608
	Sig.	.858	.819	.715	.950	.771	.257	.449	.529	.258	.148
	N	7	7	7	7	7	7	7	7	7	7
FM (kg)	**r**	.179	-.249	-.242	-.111	-.134	-.618	-.326	.126	-.502	.730
	Sig.	.701	.590	.600	.813	.774	.139	.476	.787	.251	.063
	N	7	7	7	7	7	7	7	7	7	7
FFM (kg)	**r**	.656	-.573	-.383	-.568	.165	-.327	.177	-.868*	.292	.463
	Sig.	.109	.179	.396	.183	.724	.475	.704	.011	.525	.296
	N	7	7	7	7	7	7	7	7	7	7

** - Correlation is significant at the 0.01 level (2-tailed);

* - Correlation is significant at the 0.05 level (2-tailed).

ALT: alanine transaminase, **AST:** aspartate transaminase, **BMI**: body mass index, **CK**: creatine kinase, **FM**: fat mass, **FFM**: free fat mass, **HDL**: high-density lipoprotein, **LDH**: lactate dehydrogenase, **LDL**: low-density lipoprotein, **TChol**: total cholesterol, **TG**: triglycerides.

Blue: lean group's correlations. **Green**: athlete group's correlations.

Regarding body composition and biochemistry parameters in the obese group, a moderate positive linear correlation was found between BMI and glucose (r=0.759), BMI and ALT (r=0.744) and between FM and TG (r=0.758) (**Table 9**).

Table 9 – Pearson Correlation Coefficient (r) with p-value between the body composition and the biochemistry parameters in the obese group.

OBESE GROUP		Glucose (mg/dL)	TChol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)	AST (U/L)	ALT (U/L)	LDH (U/L)	CK (U/L)	Albumin (g/dL)
Height (cm)	**r**	-.150	-.646	-.253	-.624	-.160	-.179	.187	-.449	-.587	.338
	Sig.	.723	.083	.545	.098	.706	.671	.658	.265	.126	.412
	N	8	8	8	8	8	8	8	8	8	8
Weight (kg)	**r**	.480	-.154	-.406	-.167	.066	-.026	.637	-.401	-.181	.179
	Sig.	.229	.716	.318	692	.877	.951	.089	.325	.668	.671
	N	8	8	8	8	8	8	8	8	8	8
BMI (kg/m2)	**r**	.759*	.290	-.407	.262	.188	.077	.744*	-.249	.198	-.049
	Sig.	.029	.485	.317	.530	.656	.856	.034	.552	.638	.909
	N	8	8	8	8	8	8	8	8	8	8
FM (%)	**r**	.212	.073	.184	-.106	.758*	-.209	-.046	-.051	.168	.071
	Sig.	.615	.864	.663	.803	.029	.619	.914	.905	.691	.868
	N	8	8	8	8	8	8	8	8	8	8
FM (kg)	**r**	.572	-.031	-.178	-.189	.687	-.191	.480	-.361	.010	.182
	Sig.	.139	.943	.673	.654	.060	.650	.229	.380	.982	.667
	N	8	8	8	8	8	8	8	8	8	8
FFM (kg)	**r**	.242	-.167	-.385	-0.90	-.324	.081	.486	-.271	-.223	.109
	Sig.	.563	.692	.347	.832	.433	.849	.222	.516	.595	.797
	N	8	8	8	8	8	8	8	8	8	8

* - Correlation is significant at the 0.05 level (2-tailed).

ALT: alanine transaminase, **AST:** aspartate transaminase, **BMI**: body mass index, **CK**: creatine kinase, **FM**: fat mass, **FFM**: free fat mass, **HDL**: high-density lipoprotein, **LDH**: lactate dehydrogenase, **LDL**: low-density lipoprotein, **TChol**: total cholesterol, **TG**: triglycerides.

Blue: lean group's correlations. **Green**: athlete group's correlations. **Red**: obese group's correlations.

4.6.2. Correlations between body composition parameters and protein levels

The correlation between body composition parameters and protein levels was also studied. Regarding to the lean group no correlations were found between these parameters (**Tables 10**).

Table 10 – Pearson Correlation Coefficient (r) with p-value between the body composition parameters and the serum proteins in the lean group.

LEAN GROUP		IL-6 (AU)	CRP (AU)	Myostatin (AU)	TWEAK (AU)
Height (cm)	**r**	.476	.187	.384	.478
	Sig	.281	.688	.395	.278
	N	7	7	7	7
Weight (kg)	**r**	.627	.276	.518	.111
	Sig	.132	.549	.233	.813
	N	7	7	7	7
BMI (kg/m2)	**r**	.494	.234	.421	-.038
	Sig	.260	.614	.347	.935
	N	7	7	7	7
FM (%)	**r**	.706	.165	.722	-.036
	Sig	.076	.723	.067	.938
	N	7	7	7	7
FM (kg)	**r**	.723	.244	.664	.068
	Sig	.066	.598	.104	.884
	N	7	7	7	7
FFM (kg)	**r**	.514	.287	.369	.138
	Sig	.238	.532	.415	.769
	N	7	7	7	7

BMI: body mass index**; CRP**: C-reactive protein. **FM**: fat mass; **FFM**: free fat mass; **IL-6**: interleukin 6; **TWEAK**: tumor necrosis factor weak inducer of apoptosis.
Blue: lean group's correlations.

Regarding to the athlete group, also no correlations were found between the body composition parameters and the serum proteins levels (**Table 11**).

Table 11 – Pearson Correlation Coefficient (r) with p-value between the body composition parameters and the serum proteins in the athlete group.

ATHLETE GROUP		IL-6 (AU)	CRP (AU)	Myostatin (AU)	TWEAK (AU)
Height (cm)	R	-.033	.167	-.278	.348
	Sig.	.938	.693	.505	.398
	N	8	8	8	8
Weight (kg)	**r**	.031	.068	-.347	.152
	Sig.	.943	.873	.400	.720
	N	8	8	8	8
BMI (kg/m2)	**r**	.063	-.127	-.303	-.204
	Sig.	.883	.765	.466	.628
	N	8	8	8	8
FM (%)	**r**	.116	.094	.029	-.300
	Sig.	.805	.840	.950	.513
	N	7	7	7	7
FM (kg)	**r**	.130	.171	.029	-.197
	Sig.	.780	.713	.951	.672
	N	7	7	7	7
FFM (kg)	**r**	-.226	.064	-.356	.395
	Sig.	.627	.892	.433	.380
	N	7	7	7	7

BMI: body mass index**; CRP**: C-reactive protein. **FM**: fat mass; **FFM**: free fat mass; **IL-6**: interleukin 6; **TWEAK**: tumor necrosis factor weak inducer of apoptosis.
Green: athlete group's correlations.

In the obese group, a moderate negative linear correlation between FM and TWEAK (r=-0.785) was observed (**Table 12**).

Table 12 – Pearson Correlation Coefficient (r) with p-value between the body composition parameters and the serum proteins in the obese group.

OBESE GROUP		IL-6 (AU)	CRP (AU)	Myostatin (AU)	TWEAK (AU)
Height (cm)	**r**	.079	-.416	-.217	-.478
	Sig.	.853	.305	.606	.231
	N	8	8	8	8
Weight (kg)	**r**	.318	-.012	-.133	-.397
	Sig.	.443	.977	.753	.330
	N	8	8	8	8
BMI (kg/m2)	**r**	.417	.320	.031	-.173
	Sig.	.304	.440	.942	.683
	N	8	8	8	8
FM (%)	**r**	-.335	.070	-.415	-.580
	Sig.	.417	.869	.306	.132
	N	8	8	8	8
FM (kg)	**r**	-.018	.065	-.426	-.785*
	Sig.	.967	.879	.292	.021
	N	8	8	8	8
FFM (kg)	**r**	.393	-.053	.090	-.017
	Sig.	.335	.901	.832	.969
	N	8	8	8	8

* - Correlation is significant at the 0.05 level (2-tailed).
BMI: body mass index**; CRP**: C-reactive protein. **FM**: fat mass; **FFM**: free fat mass; **IL-6**: interleukin 6; **TWEAK**: tumor necrosis factor weak inducer of apoptosis.
Red: obese group's correlations.

4.6.3. Correlations between biochemistry parameters and protein levels

The correlation between the biochemistry parameters and the protein levels in the three groups of children was studied. In the lean group a moderate negative linear correlation between AST and CRP was found (r=-0.792) (**Table 13**).

Table 13 – Pearson Correlation Coefficient (r) with p-value between the biochemistry parameters and the serum proteins in the lean group.

LEAN GROUP		IL-6 (AU)	CRP (AU)	Myostatin (AU)	TWEAK (AU)
Glucose (mg/dL)	**r**	-.622	-.347	-.691	.143
	Sig.	.099	.400	.058	.736
	N	8	8	8	8
TChol (mg/dL)	**r**	.056	-.063	.269	-.377
	Sig.	.896	.883	.520	.357
	N	8	8	8	8
HDL (mg/dL)	**r**	-.191	-.055	-.127	.102
	Sig.	.650	.896	.764	.810
	N	8	8	8	8
LDL (mg/dL)	**r**	.009	-.130	.221	-.531
	Sig.	.983	.759	.599	.176
	N	8	8	8	8
TG (mg/dL)	**r**	.590	.288	.669	-.019
	Sig.	.123	.489	.069	.964
	N	8	8	8	8
AST (U/L)	**r**	-.475	-.792*	-.239	-.116
	Sig.	.235	.019	.569	.785
	N	8	8	8	8
ALT (U/L)	**r**	-.125	-.626	.069	.080
	Sig.	.769	.097	.872	.851
	N	8	8	8	8
LDH (U/L)	**r**	-.102	-.102	.094	-.455
	Sig.	.810	.810	.824	.257
	N	8	8	8	8
CK (U/L)	**r**	.339	.077	.160	-.133
	Sig.	.412	.856	.705	.754
	N	8	8	8	8
Albumin (g/dL)	**r**	-.213	-.656	.028	-.117
	Sig.	.613	.077	.948	.784
	N	8	8	8	8

* - Correlation is significant at the 0.05 level (2-tailed).

ALT: **alanine transaminase,** AST: **aspartate transaminase,** CK: **creatine kinase,** CRP: **C-reactive protein,** HDL: **high-density lipoprotein,** IL-6: **interleukin 6,** LDH: **lactate dehydrogenase,** LDL: **low-density lipoprotein,** TChol: **total cholesterol,** TG: **triglycerides**; **TWEAK**: tumor necrosis factor weak inducer of apoptosis.

Blue: lean group's correlations.

In the athlete group a moderate negative linear correlation between glucose and Myostatin (r=0.799) was observed (**Table 14**).

Table 14 – Pearson Correlation Coefficient (r) with p-value between the biochemistry parameters and the serum proteins in the athlete group.

ATHLETE GROUP		IL-6 (AU)	CRP (AU)	Myostatin (AU)	TWEAK (AU)
Glucose (mg/dL)	**r**	-.293	-.467	-.799*	-.205
	Sig.	.481	.243	.017	.627
	N	8	8	8	8
TChol (mg/dL)	**r**	-.555	-.446	-.037	-.480
	Sig.	.154	.268	.931	.229
	N	8	8	8	8
HDL (mg/dL)	**r**	-.487	-.413	.005	-.516
	Sig.	.221	.309	.991	.191
	N	8	8	8	8
LDL (mg/dL)	**r**	-.621	-.395	.003	-.503
	Sig.	.101	.333	.994	.204
	N	8	8	8	8
TG (mg/dL)	**r**	.493	.106	-.172	.504
	Sig.	.215	.803	.685	.203
	N	8	8	8	8
AST (U/L)	**r**	-.561	-.362	.024	-.353
	Sig.	.148	.378	.955	.391
	N	8	8	8	8
ALT (U/L)	**r**	-.345	.324	.423	.390
	Sig.	.402	.433	.296	.339
	N	8	8	8	8
LDH (U/L)	**r**	-.122	-.468	-.043	-.699
	Sig.	.773	.242	.919	.054
	N	8	8	8	8
CK (U/L)	**r**	-.082	-.164	-.252	.382
	Sig.	.848	.697	.548	.350
	N	8	8	8	8
Albumin (g/dL)	**r**	.253	.319	-.146	.237
	Sig.	.545	.441	.730	.571
	N	8	8	8	8

* - Correlation is significant at the 0.05 level (2-tailed).

ALT: alanine transaminase, **AST:** aspartate transaminase, **CK:** creatine kinase, **CRP:** C-reactive protein, **HDL:** high-density lipoprotein, **IL-6:** interleukin 6, **LDH:** lactate dehydrogenase, **LDL:** low-density lipoprotein, **TChol:** total cholesterol, **TG:** triglycerides**;** TWEAK**: tumor necrosis factor weak inducer of apoptosis.**

Blue: lean group's correlations. **Green**: athlete group's correlations.

Regarding to the obese group a moderate positive linear correlation was found between TChol and CRP (r=0.710), and a strong positive linear correlation was observed between CK and CRP (r=0.879). A moderate negative linear correlation was also found between Albumin with IL-6 (r=-0.797) and Myostatin (r=-0.783), while a strong negative linear correlation was found between Albumin and CRP (r=-0.886) (**Table 15**).

Table 15 – Pearson Correlation Coefficient (r) with p-value between the biochemistry parameters and the serum proteins in the obese group.

OBESE GROUP		IL-6 (AU)	CRP (AU)	Myostatin (AU)	TWEAK (AU)
Glucose (mg/dL)	**r**	-.128	-.054	-.219	-.254
	Sig.	.763	.899	.603	.545
	N	8	8	8	8
TChol (mg/dL)	**r**	.260	.710*	.296	.426
	Sig.	.534	.048	.476	.293
	N	8	8	8	8
HDL (mg/dL)	**r**	-.364	-.202	-.595	.193
	Sig.	.375	.631	.120	.647
	N	8	8	8	8
LDL (mg/dL)	**r**	.332	.691	.482	.546
	Sig.	.422	.058	.227	.162
	N	8	8	8	8
TG (mg/dL)	**r**	-.264	.168	-.694	-.487
	Sig.	.528	.691	.056	.221
	N	8	8	8	8
AST (U/L)	**r**	-.215	-.112	-.417	.333
	Sig.	.608	.791	.304	.420
	N	8	8	8	8
ALT (U/L)	**r**	.403	.144	-.127	-.052
	Sig.	.322	.734	.765	.902
	N	8	8	8	8
LDH (U/L)	**r**	-.312	-.122	-.398	.479
	Sig.	.452	.773	.329	.230
	N	8	8	8	8
CK (U/L)	**r**	.351	.879**	.239	.393
	Sig.	.394	.004	.568	.335
	N	8	8	8	8
Albumin (g/dL)	**r**	-.797*	-.886**	-.783*	-.500
	Sig.	.018	.003	.022	.207
	N	8	8	8	8

** - Correlation is significant at the 0.01 level (2-tailed);

* - Correlation is significant at the 0.05 level (2-tailed).

ALT: alanine transaminase, **AST:** aspartate transaminase, **CK:** creatine kinase, **CRP:** C-reactive protein, **HDL:** high-density lipoprotein, **IL-6:** interleukin 6, **LDH:** lactate dehydrogenase, **LDL:** low-density lipoprotein, **TChol:** total cholesterol, **TG:** triglycerides**;** TWEAK**: tumor necrosis factor weak inducer of apoptosis.**

Blue: lean group's correlations. **Green**: athlete group's correlations. **Red**: obese group's correlations.

5. Discussion

Obesity results from an excess of adipose tissue and is recongnized as a health problem which has reached epidemic proportions worldwide. Childhood obesity is even a more dramatic issue being connected to adverse consequences that remain to adulthood and are associated with increased cardiovascular problems. Inflammation and oxidative stress are closely linked to childhood obesity triggering endothelial dysfunction [1,9]. Physical exercise contributes to the regulation of adipose tissue and is essential to treat and prevent obesity. In order to promote health and quality of life, the individuals should embrace regular moderate exercise in a routine base [61,71]. However, the number of children who practice intense exercise at an early age is being increasing [84]. Intense training and competition can cause adverse consequences not only for the body systems but also psychologically [82,84]. Due to these factors is important to understand how the obesity as well as the intense exercise affect the physical and physiological parameters in children.

Several anthropometric measures of body composition were analyzed in lean, athlete and obese children considering the age, gender and the kind of sport practiced. Among the obese children studied, weight, BMI and fat mass (FM) are statistically higher being in agreement with previous data [92]. Since the average of age is substantially high in the obese group, is important to consider bone mass and organs growth as a factor for heavier body weight in this group. This aspect also influence the body mass index (BMI – kg/m^2), an anthropometric tool to assess relative weight being the most commonly used to classify obesity in children [92]. In addition to an excess of fat mass, high BMI is frequently associated with metabolic changes, increased prevalence of cardiovascular disease and inflammation [93]. In our study, BMI is positively correlated with glucose and ALT suggesting that, in childhood obesity, a high BMI could be associated with the development of type-2 diabetes mellitus and liver injury [94,95]. The weight gain in obesity induces insulin resistance decreasing the insulin-sensitive receptors leading to an increase in blood glucose level due to delayed glucose uptake. These alterations are normally associated with increased serum activity of liver enzymes such as ALT,

contributing to diabetes and hepatic disease [94]. However, AST levels, another biomarker for liver health [96], are statistically diminished in obese children but within the reference values, which can suggest that obesity may not be associated with liver disease in children contrary to what happens in obese adults. In other hand, the obese children studied have increased levels of LDH, above the reference values. High levels of this enzyme in the blood suggest cell damage and tissues/organ illness (cardiac and skeletal muscles, liver, red cells, lungs or kidney) [97]. However, this does not suggest hepatic injury since AST is more liver-specific than LDH.

Another positive correlation found in obese children was between fat mass and triglycerides contributing to the typical dyslipidemia of obesity. Unfavorable lipid levels were common among obese children and adolescents [98]. The changes in the lipid profile may be accentuated in the obese group since it includes boys but also female adolescents who probably menstruate. While HDL levels were decreased in the obese group, LDL levels are increased and both are outside the reference values. Due to this, the uptake of cholesterol from peripheral tissues to the liver by HDL is decreased while high amount of lipid molecules are transported by LDL into the arterial walls contributing for arteriosclerosis [99].

Creatine kinase (CK) plays a key role in cellular energy metabolism being higher in skeletal muscle type II fibers. This promotes the storage of fatty acids and glucose as lipids in adipose tissue instead of the uptake and oxidation in skeletal muscle, which may lead to obesity. CK is strongly and independently associated with body mass and high levels are related to muscle damage [100]. In this study this correlation was found in the lean group, where CK was also related with free-fat mass (FFM). In that case, the increase of BMI is due to the high FFM being positively correlated with CK [100]. Our results are the expected since the obese group has low levels of CK in comparison to the lean and the athlete groups.

All the lipid abnormalities reported previously are typical features of childhood obesity and may be associated to a pro-inflammatory state which may start in the adipose tissue and directly affect the endothelium [55]. In fact, high levels of inflammatory markers (CRP, IL-6 and TWEAK) were found in the obese children of this study. C-reactive protein is positively associated with total cholesterol and CK. Myostatin levels were also increased in the serum of the obese participants. This increase was previously

shown by Zhu et al. and are related with decreased skeletal muscle mass [52]. Myostatin as well as Il-6 and CRP are negatively correlated with Albumin suggesting that inflammation is associated with reduced Albumin synthesis or increased catabolic rate. This could lead to hypoalbuminemia which in turn may be related to liver disease since Albumin is a serum hepatic protein [101].

Inadequate energy and nutrient balance may occur among childhood athletes, which can result in delayed pubertal development and retarded growth [102]. Our study is consistent with the previous data since the athlete group has children with low weight, BMI and fat mass and high free-fat mass when compared to the lean and obese groups. Weight is negatively associated with lipids (TChol and LDL) in children who practice intense sports since they have high levels of free-fat mass contributing for their weight instead of fat mass. Eisenmann et al. as well as Evangelos et al. suggested similar levels of TChol, lower levels of TG and LDL, and higher levels of HDL in childhood athletes compared with controls. They concluded that childhood athletes have a better blood lipid profile then children who do not participate in any extra exercise except from school sport activities [103,104]. Our study shows the same alterations in the lipid profile of athlete children but these benefits were not significant.

Elevated levels of AST are specifically associated with muscular injury like myocardial infarction or myolysis, and with athletic activity [105]. CK and LDH also increase considerable after intensive exercise and in muscle pathology. Both of these enzymes indicate the degree of metabolic adaptations of skeletal muscle to physical training [106]. The athlete group shows increased levels of AST, CK and LDH regarding to the lean group being in accordance with previous data. However, these results did not reveal statistical significance.

In order to ensure a proper growth, development and maturation of athlete children, an adequate energy intake is needed. Younger athletes usually depend more on fat as a fuel and have smaller glycogen stores and a limited glycolytic capacity. During strenuous exercise, glucose is important for contracting skeletal muscle being its uptake determined by exercise intensity and duration. Exercise training is also the most potent stimulus to increase skeletal muscle GLUT4 expression contributing for glucose uptake [107,108]. Athlete children in this study have low levels of glucose in the blood, even when compared with the lean group, which strengthens the previous data. The levels of glucose in the

blood are negatively correlated with Myostatin. There is evidence that physical exercise regulates Myostatin improving glucose homeostasis and consequently enhancing insulin sensitivity [109]. However, we found low levels of glucose and high, but not significant, levels of Myostatin in the serum of the athletes, suggesting that the increase of Myostatin may be related to weight gain in terms of free-fat mass in athletes.

The immune system and the cytokine response of the athletes could be deeply modified by exercise performed at a competitive level [110]. The athletes studied reveal increased levels of pro-inflammatory markers in serum such as IL-6, CRP and TWEAK in comparison with children of the control group. Nevertheless, these results did not demonstrate statistical significance, excluding the hypothesis of inflammation caused by intense exercise in children. Regarding to swimmers, the increase of pro-inflammatory cytokines appeared to be directly linked to the intensity and duration of the exercise in combination with exposure to chlorination's products [80]. However, according to our results, the practice of competitive swimming by children does not seem to be harmfull in terms of inflammation.

During exercise an efficient transport of oxygen within the body is essential to supply working muscle maximizing the aerobic power and contributing to fatigue resistance. Red blood cells as well as hemoglobin contribute to the transport of oxygen to the tissues and improve blood flow to working muscle. Strenuous exercise is also characterized by an iron-deficiency anemia resulting from a low iron intake or caused by exercise itself [111,112]. Regarding to the athlete group, the MCHC, which indicates the concentration of hemoglobin in red blood cells, is statistically low but it is within the reference values.

According to the results obtained, intense physical exercise in children may contribute to the development of healthy lifestyles and may start earlier in life, while obesity is associated with poor outcomes.

6. Conclusions and future prospects

6.1. Conclusions

Body composition, lipid profile and protein levels mainly related with inflammation provided evidence supporting that childhood obesity is detrimental. Children with high levels of body fat reveal atypical lipid profile (low HDL and high LDL), tissue damage due to the high levels of LDH, and also chronic inflammation (high IL-6, CRP and TWEAK). These alterations link obesity to atherosclerosis and to cardiovascular diseases in long-term. However, the low levels of hepatic enzymes such as AST and ALT do not associate obesity with hepatic damage. Our results confirmed that childhood obesity is a major health problem being important its prevention. Regular exercise in conjunction with normal calorie intake are key factors for weight loss in children reducing inflammatory activation and also preventing vascular disease.

Regarding the athletes, the intense physical exercise showed benefits to the health of children since it mainly decreased the levels of blood glucose protecting children from diseases like diabetes. Children who practice intense exercise also reveal, but without statistical significance, a healthy body composition with high levels of free-fat mass instead of fat and an improved lipid profile. This type of exercise does not seem to be related with inflammation once the levels of pro-inflammatory cytokines are similar in both lean and athlete groups. These results confirm that physical activity must start in early life since it contributes to the development of healthy lifestyles. However, the definition of the optimal intensity and duration of exercise in children is important to avoid the potencial risks from early excessive training.

6.2. Future prospects

An important future goal will be to have homogenous groups only with male participants aged between 12 and 15 years old. It is also important to recruit more participants to each group in order to have a sample size which adequately represents the population of obese and athlete children. The same physical and biological parameters should be evaluated in the three groups of children studied.

Considerable progress should be expected if a group of trained obese children will be inserted in the study. This will allow studying the impact of exercise in childhood obesity.

Future research should also focus on the evaluation of cardiorespiratory fitness through shuttle-run test, cardiac function by echocardiography, vascular function through the assessment of endothelium-dependent vasodilation and the evaluation of blood pressure. Another interesting evaluation should be to estimate the oxidative stress and the endothelial repair/regeneration. With these improvements, it will be possible to know if the children studied suffer from endothelial dysfunction and cardiovascular disease.

Studing the signaling pathways involved in childhood obesity as well as in exercise practiced by children will allow the understanding of the molecular mechanisms involved in each case and if there are commonalities.

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8. Appendix

8.1. Questionnaire

Q1: Gender.

Q2: Do you do sports activities outside of school?

Q3: In addition to the teaching acivities, how often do you do physical activity or sport at least 20 minutes?

Q4: Outside of school time, how much time do you dedicate practicing physical activity and sport until became breathless (breathing rapidly or with difficulty) and sweaty?

Q5: Do you participate in sports competitions?

Q6: If you answered to i) (playing a sport oriented by a coach/teacher) refere which sport.

8.2. Results from immuno-blot assay

Table 16 – Results from the immune-blot assay.

	IL-6			CRP			Myostatin			TWEAK		
	S	**C**	**R**	**S**	**C**	**R**	**S**	**C**	**R**	**S**	**C**	**R**
L1	444.59	18.98	23.43	695.78	22.48	30.95	680.14	29.80	22.83	702.74	27.69	25.38
L2	475.57	19.29	24.65	720.82	22.10	32.62	708.60	30.43	23.29	721.83	28.05	25.74
L3	442.80	19.18	23.09	683.91	21.55	31.73	675.28	30.26	22.31	693.12	27.79	24.95
L4	458.76	18.97	24.19	705.02	21.42	32.91	697.44	30.23	23.07	742.58	28.08	26.45
L5	454.03	18.93	23.98	700.59	21.36	32.80	700.13	30.41	23.02	756.73	28.03	27.00
L6	463.39	19.29	24.03	689.24	21.38	32.24	694.80	30.33	22.91	748.24	29.13	25.68
L7	472.26	19.78	23.87	694.64	21.63	32.12	689.90	29.95	23.04	771.68	30.82	25.04
L8	486.39	20.03	24.28	706.01	21.51	32.82	697.38	30.01	23.24	781.88	31.49	24.83
A1	479.60	20.35	23.56	689.84	21.31	32.38	693.27	30.38	22.82	759.13	30.80	24.65
A2	481.79	20.51	23.50	705.10	21.33	33.06	674.57	28.57	23.61	801.14	30.31	26.43
A3	483.37	20.28	23.84	702.02	21.21	33.10	678.67	28.78	23.59	770.44	29.89	25.78
A4	485.93	19.57	24.83	694.15	21.00	33.06	684.98	28.33	24.18	763.12	28.96	26.35
A5	495.28	19.49	25.41	726.91	22.01	33.02	709.32	31.02	22.87	764.44	27.90	27.40
A6	456.43	20.50	22.27	685.03	21.61	31.70	700.16	31.98	21.89	704.97	28.05	25.14
A7	530.07	22.35	23.72	734.41	21.78	33.72	733.58	30.63	23.95	798.58	28.83	27.70
A8	520.20	20.89	24.90	718.46	21.43	33.52	721.62	30.67	23.53	803.71	30.19	26.62
O1	526.12	20.68	25.44	740.77	21.71	34.12	730.13	31.43	23.23	829.00	31.27	26.51
O2	526.86	20.83	25.29	742.51	21.91	33.89	728.45	30.91	23.57	836.97	31.51	26.56
O3	569.36	21.85	26.06	776.19	22.23	34.91	758.67	31.81	23.85	890.82	32.37	27.52
O4	519.88	22.30	23.31	731.05	21.86	33.44	724.47	30.82	23.51	830.04	31.10	26.69
O5	554.92	20.81	26.67	739.64	21.65	34.17	744.32	29.76	25.01	814.30	30.28	26.89
O6	561.81	20.90	26.88	753.65	21.77	34.62	755.60	29.41	25.69	828.81	30.75	26.95
O7	537.84	20.77	25.90	738.97	21.18	34.89	733.29	29.08	25.21	803.59	30.03	26.76
O8	547.76	20.72	26.43	724.72	21.07	34.40	721.07	27.72	26.01	805.69	28.68	28.09

L1-L8: lean group, **A1-A8**: athlete group, **O1-O8**: obese group, **S**: sample, **C**: control, **R**: ratio.

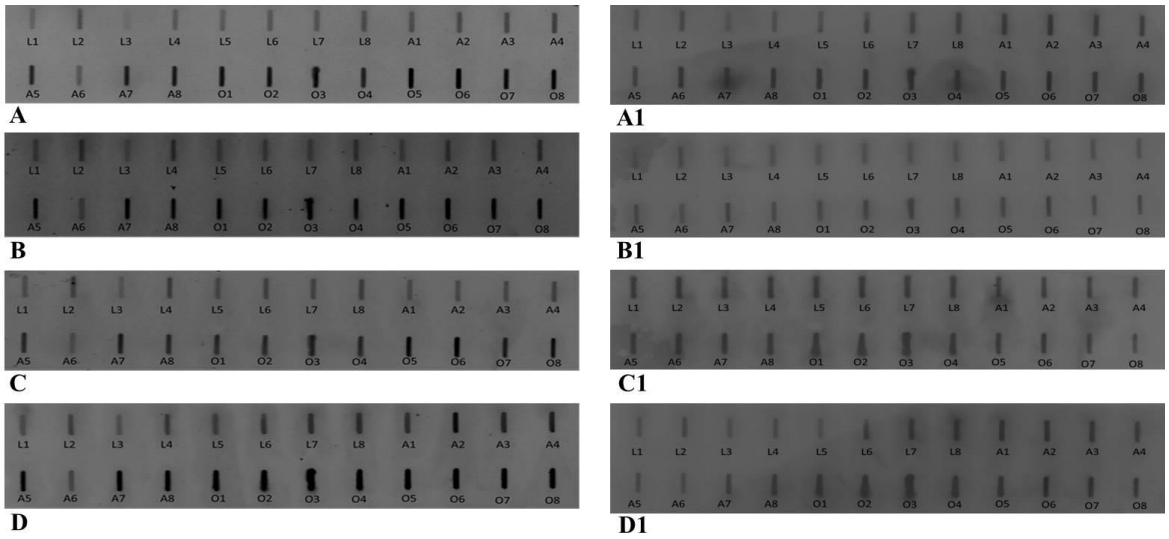

Figure 7 – Protein levels [**(A)** IL-6, **(B)** CRP, **(C)** Myostatin, **(D)** TWEAK] in serum samples from lean (L1-L8), athlete (A1-A8) and obese (O1-O8) groups and their respective ponceau controls [**(A1)** IL-6, **(B1)** CRP, **(C1)** Myostatin, **(D1)** TWEAK].

8.3. Statistical results – Hypothesis tests

8.3.1. Body composition parameters

Table 17 – Statistical tests for comparing two independent samples (Lean/Athlete, Lean/Obese and Athlete/Obese) in relation to the body composition parameters.

Variable	Study Groups		N	Test	Statistical test	p-value (2-tailed)	Significantly higher
Height (cm)	Lean	Athlete	7	Independent sample t-test[a)]	t=0.967	0.35	-
		Obese	7	Independent sample t-test[a)]	t=0.353	0.73	-
	Athlete	Obese	8	Independent sample t-test[a)]	t=-0.707	0.491	
Weight (kg)	Lean	Athlete	7	Independent sample t-test[a)]	t=1.881	0.12	-
		Obese	7	Independent sample t-test[a)]	t=-3.739	0.002*	Obese
	Athlete	Obese	8	Independent sample t-test[a)]	t=-6.333	0.00*	Obese
BMI (kg/m2)	Lean	Athlete	7	Independent sample t-test[a)]	t=1.508	0.16	-
		Obese	7	Independent sample t-test[a)]	t=-4.777	0.00*	Obese
	Athlete	Obese	8	Independent sample t-test[a)]	t=-8.781	0.00*	Obese
FM (%)	Lean	Athlete	7	Independent sample t-test[a)]	t=1.289	0.22	-
		Obese	7	Independent sample t-test[a)]	t=-6.755	0.00*	Obese
	Athlete	Obese	7	Independent sample t-test[a)]	t=-7.959	0.00*	Obese
FM (kg)	Lean	Athlete	7	Independent sample t-test[a)]	t=1.550	0.15	-
		Obese	7	Independent sample t-test[a)]	t=-7.155	0.00*	Obese
	Athlete	Obese	7	Independent sample t-test[a)]	t=-9.851	0.00*	Obese
FFM (kg)	Lean	Athlete	7	Independent sample t-test[a)]	t=0.861	0.41	-
		Obese	7	Independent sample t-test[a)]	t=-0.465	0.648	-
	Athete	Obese	7	Independent sample t-test[a)]	t=-1.192	0.255	-

[a)] – Independent sample t-test. Equal variances assumed (Levene´s test for equality of variables – p-value>0.05);

* - The difference is significant at the 0.05 level;

BMI: body mass index, **FM**: fat mass, **FFM**: free-fat mass.

8.3.2. Hemogram parameters

Table 18 – Statistical tests for comparing two independent samples (Lean/Athlete) in relation to the hemogram parameters.

Variable	Study Groups		N	Test	Statistical test	p-value (2-tailed)	Significantly higher
Erythrocytes (10^12/L)	Lean	Athlete	8	Independent sample t-test[a)]	t=-0.146	0.89	-
Hb (g/dL)	Lean	Athlete	8	Mann-Whitney test[c)]	U=29; W=65	0.80	-
Hematocrit (%)	Lean	Athlete	8	Mann-Whitney test[c)]	U=31; W=67	0.96	-
MCV (fL)	Lean	Athlete	8	Mann-Whitney test[c)]	U=19; W=55	0.20	-
MCH (pg)	Lean	Athlete	8	Mann-Whitney test[c)]	U=31.5; W=67.5	0.96	-
MCHC (g/dL)	Lean	Athlete	8	Independent sample t-test[a)]	t=3.052	0.009 *	Lean
RDW (%)	Lean	Athlete	8	Mann-Whitney test[c)]	U=21.5; W=57.5	0.28	-
Leukocytes (10^9/L)	Lean	Athlete	8	Independent sample t-test[a)]	t=0.243	0.81	-
Neutrophils (%)	Lean	Athlete	8	Independent sample t-test[a)]	t=-1.982	0.07	-
Eosinophils (%)	Lean	Athlete	8	Independent sample t-test[b)]	t=2.076	0.07	-
Basophils (%)	Lean	Athlete	8	Mann-Whitney test[c)]	U=31.5; W=67.5	0.96	-
Lymphocytes (%)	Lean	Athlete	8	Independent sample t-test[a)]	t=0.142	0.89	-
Monocytes (%)	Lean	Athlete	8	Independent sample t-test[a)]	t=-1.351	0.20	-
Platelets (10^9/L)	Lean	Athlete	8	Independent sample t-test[a)]	t=-0.033	0.97	-
MPV (fL)	Lean	Athlete	8	Independent sample t-test[a)]	t=-1.480	0.16	-
PDW (fL)	Lean	Athlete	8	Independent sample t-test[a)]	t=-0.840	0.42	-

[a)] – Independent sample t-test. Equal variances assumed (Levene´s test for equality of variables – p-value>0.05);

[b)] – Independent sample t-test. Equal variances not assumed (Levene´s test for equality of variables – p-value<0.05);

[c)] – Mann-Whitney test.

* - The difference is significant at the 0.05 level;

Hb: haemoglobin, **MCH**: mean corpuscular hemoglobin, **MCHC**: mean corpuscular hemoglobin concentration, **MCV**: mean corpuscular volume, **MPV**: mean platelet volume, **PDW**: platelet distribution width, **RDW**: red cell distribution width.

8.3.3. Biochemistry parameters

Table 19 – Statistical tests for comparing two independent samples (Lean/Athlete, Lean/Obese and Athlete/Obese) in relation to the biochemistry parameters.

Variable	Study Groups		N	Test	Statistical test	p-value (2-tailed)	Significantly higher
Glucose (mg/dL)	Lean	Athlete	8	Independent sample t-test[b)]	t=4.526	0.00*	Lean
		Obese	8	Independent sample t-test[a)]	t=0.714	0.49	-
	Athlete	Obese	8	Independent sample t-test[a)]	t=-2.026	0.06	-
TChol (mg/dL)	Lean	Athlete	8	Independent sample t-test[a)]	t=-0.125	0.90	-
		Obese	8	Independent sample t-test[a)]	t=-2.365	0.03	-
	Athlete	Obese	8	Independent sample t-test[a)]	t=-1.905	0.08	-
HDL (mg/dL)	Lean	Athlete	8	Independent sample t-test[a)]	t=-1.198	0.25	-
		Obese	8	Independent sample t-test[b)]	t=10.558	0.00*	Lean
	Athlete	Obese	8	Independent sample t-test[b)]	t=9.534	0.00*	Athlete
LDL (mg/dL)	Lean	Athlete	8	Independent sample t-test[a)]	t=0.424	0.68	-
		Obese	8	Independent sample t-test[a)]	t=-6.312	0.00*	Obese
	Athlete	Obese	8	Independent sample t-test[a)]	t=-6.214	0.00*	Obese
TG (mg/dL)	Lean	Athlete	8	Mann-Whitney test[c)]	U=28; W=64	0.72	-
		Obese	8	Independent sample t-test[a)]	t=0.777	0.30	-
	Athlete	Obese	8	Mann-Whitney test[c)]	U=15; W=51	0.08	-
AST (U/L)	Lean	Athlete	8	Mann-Whitney test[c)]	U=15; W=51	0.08	-
		Obese	8	Mann-Whitney test[c)]	U=3; W=39	0.001*	Lean
	Athlete	Obese	8	Independent sample t-test[a)]	t=8.739	0.00*	Athlete
ALT (U/L)	Lean	Athlete	8	Mann-Whitney test[c)]	U=26; W=62	0.57	-
		Obese	8	Mann-Whitney test[c)]	U=31; W=67	0.96	-
	Athlete	Obese	8	Independent sample t-test[a)]	t=0.738	0.47	-
LDH (U/L)	Lean	Athlete	8	Independent sample t-test[a)]	t=-0.252	0.81	-
		Obese	8	Independent sample t-test[b)]	t=-2.570	0.02 *	Obese
	Athlete	Obese	8	Independent sample t-test[b)]	t=-2.471	0.03*	Obese
CK (U/L)	Lean	Athlete	8	Independent sample t-test[a)]	t=-2.122	0.052	-
		Obese	8	Independent sample t-test[b)]	t=4.765	0.00*	Lean
	Athlete	Obese	8	Independent sample t-test[b)]	t=8.940	0.00*	Athlete
Albumin (g/dL)	Lean	Athlete	8	Mann-Whitney test[c)]	U=11; W=47	0.03	-
		Obese	8	Mann-Whitney test[c)]	U=24.5; W=60.5	0.44	-
	Athlete	Obese	8	Independent sample t-test[b)]	t=-1.514	0.152	-

[a)] – Independent sample t-test. Equal variances assumed (Levene´s test for equality of variables – p-value>0.05); [b)] – Independent sample t-test. Equal variances not assumed (Levene´s test for equality of variables – p-value<0.05); [c)] – Mann-Whitney test.

* - The difference is significant at the 0.05 level;

ALT: alanine transaminase, **AST**: aspartate transaminase, **CK**: creatine kinase, **HDL**: high density lipoprotein, **LDH**: lactate dehydrogenase, **LDL**: low density lipoprotein, TChol: total cholesterol, TG: triglycerides.

8.3.4. Protein levels (IL-6, CRP, Myostatin and TWEAK)

Table 20 – Statistical tests for comparing two independent samples (Lean/Athlete, Lean/Obese and Athlete/Obese) in relation to the immuno-blot parameters.

Variable	Study Groups		N	Test	Statistical test	p-value (2-tailed)	Significantly higher
IL-6	Lean	Athlete	8	Independent sample t-test[a)]	t=-0.162	0.874	-
		Obese	8	Independent sample t-test[a)]	t=-4.146	0.001*	Obese
	Athlete	Obese	8	Independent sample t-test[a)]	t=-3.263	0.006*	Obese
CRP	Lean	Athlete	8	Independent sample t-test[a)]	t=-2.046	0.06	-
		Obese	8	Independent sample t-test[a)]	t=-6.824	0.00*	Obese
	Athlete	Obese	8	Independent sample t-test[a)]	t=-4.722	0.00*	Obese
Myostatin	Lean	Athlete	8	Independent sample t-test[b)]	t=-1.206	0.258	-
		Obese	8	Independent sample t-test[b)]	t=-3.859	0.005*	Obese
	Athlete	Obese	8	Independent sample t-test[a)]	t=-2.585	0.022*	Obese
TWEAK	Lean	Athlete	8	Independent sample t-test[a)]	t=1.368	0.193	-
		Obese	8	Independent sample t-test[a)]	t=-4.116	0.001*	Obese
	Athlete	Obese	8	Independent sample t-test[a)]	T=-1.774	0.098	-

[a)] – Independent sample t-test. Equal variances assumed (Levene´s test for equality of variables – p-value>0.05);

* - The difference is significant at the 0.05 level;

IL-6: interleukin 6, **CRP**: C-reactive protein, **TWEAK**: tumor necrosis factor weak inducer of apoptosis.